



Advocating the need of a systems biology approach for personalised prognosis and treatment of B-CLL patients

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Abstract

The clinical course of B-CLL is heterogeneous. This heterogeneity leads to a clinical dilemma: can we identify those patients who will benefit from early treatment and predict the survival? In recent years, mathematical modelling has contributed significantly in understanding the complexity of diseases. In order to build a mathematical model for determining prognosis of B-CLL one has to identify, characterise and quantify key molecules involved in the disease. Here we discuss the need and role of mathematical modelling in predicting B-CLL disease pathogenesis and suggest a new systems biology approach for a personalised therapy of B-CLL patients.

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1. Introduction

B-cell chronic lymphocytic leukemia (B-CLL), is the most common leukaemia in the western world. It is not curable [1] and the ability of radiation and chemotherapeutic agents to induce clinically significant regression of the disease in patients with CLL has not improved for the last decades [2]. The development of treatment approaches for B-CLL has lagged in comparison to other hematologic malignancies for various reasons. B-CLL disease seems especially prone to familial occurrence being nearly 3 times higher than that expected for the general population [3]. Historical reports exist for identical twins [4], mother and son [5], grandfather, son, and grandson [6] affected due to familial aggregation of chronic lymphocytic leukaemia pathogenesis associated with immune defects. In 1980 Conley et al [7] reported an increasing frequency

of B-CLL disease associated with autoimmune diseases, concluding that genetic factors in these families are the prime cause of deregulation in the immune system. Many reports from the Swedish family cancer database [8] evidently reported that there is significantly increased familial risk involved in B-CLL disease pathogenesis. CLL risks were similar in parents, siblings, and offspring cases, and in male and female relatives. They were not affected by the case's age at diagnosis.

Although prognostic markers have been identified, there is as yet no proven indications for initiating treatment in patients with asymptomatic disease. The lack of accuracy in predicting disease progression and survival on an individual basis has been a long asked research question in B-CLL. It's a known fact that in B-CLL

disease pathogenesis to date, no single biomarker has been useful in predicting the prognosis in CLL patients. This review focuses on our current understanding of clinical course and molecular mechanisms in B-CLL disease, and suggests the application of systems biology as an approach in personalized treatment for B-CLL patients.

2. Clinical paradigm

The clinical course of B-CLL disease is highly variable, with life expectancies ranging from months to decades. There is no apparent survival advantage for early treatment intervention, and some patients may never require treatment. At present the information available is only used to counsel patients and to help inform clinicians regarding the frequency of monitoring. The currently available clinical staging systems (e.g. Rai or Binets test) for B-CLL are simple and inexpensive but lack accuracy to predict disease progression and survival on an individual basis. Prediction was not significant when analyzed using life table methods performed by Goldin et al. [8] and it is thereby concluded that the familial component of CLL is shared with other lympho proliferative malignancies, suggesting common genetic pathways. As infection is a major cause of morbidity and mortality in B-CLL, previous studies on infection predictors in CLL were based on patients receiving chemotherapy, or on those diagnosed with infections during the 'watch and wait' period and during therapy [9-12]. Treatment strategies in preventing recurrent infection procedures in these patients, however, have not yet been devised [13].

B-CLL disease is characterized by an accumulation of mature, non-proliferating B lymphocytes in the blood, spleen, lymph nodes and bone marrow. The accumulation of B cells is the result of a proliferative defect, failing B cells to undergo apoptosis and leading to large numbers of cells being blocked in the G0/G1 phase of the cell cycle [14]. Not surprisingly, many studies have shown that B-CLL cells are resistant to various drug induced apoptosis [15]. The reasons for this are unclear, but may include a combination of various factors like reduced expression of several apoptosis inducing proteins that include receptors, kinases and enzymes.

The diagnosis of B-CLL is carried out using a number of classic and novel sensitive techniques, which allow diagnosing and differentiating the disease from other chronic lympho proliferative disorders [16-18]. Morphology and immuno phenotype are the initial diagnostic investigations, alongside molecular genetics and/or histology to exclude other B cell disorders. To date immuno phenotyping is the only method that can determine or confirm the diagnosis, as B-CLL lymphocytes carry a distinct immuno phenotypic signature (more than

5×10^9 B cells/litre). A scoring system compounding the results with a set of markers allows a refined confirmation of the diagnosis as described by Matutes et al. [18], but this scoring system cannot be applied globally to patient management, as the B-CLL disease heterogeneity exists irrespective of mutated and unmutated pattern of the IgV_H region.

B-CLL disease has been presented as two distinct types. The presence of somatic mutations in the immunoglobulin (IgH) heavy chain gene define a group of patients exhibiting stable or slowly progressive disease that requires late or no treatment. By contrast, the absence of mutations in the IgH genes of B-CLL cells define a group, which exhibit a progressive clinical course requiring early treatment [19]. Other immunological markers such as surface antigens (for example, CD38 and ZAP-70) have an important prognostic impact but still lack accurate prediction in patient survival rates and overall treatment response rates. Moreover, optimal drug administration schedules are required in order to improve clinical response in CLL patients.

3. Cell cycle, proliferation and apoptosis

B-CLL lymphocytes lack the fundamental functions of regulating the cell cycle and apoptosis. That is why the cellular factors that govern the entry of resting B-cells into the cell cycle and promote their progression through the different phases of cell cycle, are considered to be optimal targets for drug therapy. Normally the matured naive lymphocytes circulating in blood remain quiescent in the G0 phase and reside in lymphoid tissues such as the spleen until they encounter an appropriate mitogenic stimulus. The progression of the cell cycle in naive B cells depends on the PI3K activation of AKT activation, and inducing NF-κB activation and cyclin D2 activity by deregulating FOXO transcription rate [20-21]. The rate at which B lymphocytes enter the cell cycle is tightly regulated by a very complex process involving multiple components of the cell cycle machinery, apoptosis, and the antibody-generating apparatus and by downstream effectors of Bcl-2 pathway (e.g. caspases).

The proliferation rate and resistance to drug-induced apoptosis in B cells are recognized as important factors in the outcome of treatment in CLL disease. Mutated and unmutated CLL patients clearly differ in terms of prognosis. The genomic aberrations occurring in B-CLL patients have their individual effects due to distinct expression profiles, as reported by Klein et al [22]. This is termed as gene dosage effect, which is correlative with the variable mutational status of IgVh [23]. CLL cells are dependent on the continuous expression of intrinsically short lived anti-apoptotic proteins for survival. This biological context has been a promising strategy to

induce apoptosis by the inhibitors of transcription and translation. High levels of Mcl-1 and Bcl-2 mRNA [24] and protein [25] have been found in B-CLL. Mcl-1 is associated with the failure in response of B-CLL patients to initial therapy, due to its high expression [26, 27] whereas low expression of Mcl-1 in CLL cells is related to prolonged patient survival [24]. The presence of Bcl-2 (B-cell B-CLL/lymphoma 2), an anti-apoptotic protein that is highly expressed in B-CLL, and which cannot be neutralised with the levels of pro-apoptotic proteins, has been a characteristic feature for B-CLL cells when compared to normal B cells [28]. B-cell antigen receptor (BCR) is the key signalling molecule of B-lymphocytes, triggering pathways involved in B-cell proliferation, survival, differentiation, and apoptosis. In B-CLL, the status of the BCR is of important prognostic, biological, and therapeutic value. This is reflected in the great variety of emerging novel agents targeting the BCR signalling cascade.

To date large components of BCR signalling pathways have been elucidated in determining the prognosis of B-CLL, but failed to predict accurately. Pharmacological inhibition of BCR signalling in CLL can be achieved by targeting components of the BCR signalling cascade. Several groups have reported an increase in MAPK (ERK2) phosphorylation and NFAT transcriptional activity and a corresponding lack of AKT phosphorylation due to constitutive activation of distinct BCR signalling pathways in a subset of particular CLL cases [29]. The aberrant signalling mechanism in BCR signalling cascade includes the activation of PKC and PI3K pathways, which regulate B cells survival. The signal transduction inhibitors in these pathways have resulted promising results *in vitro*, but could not be used as effective single agents for B-CLL therapy. Existence of molecular heterogeneity in CLL that is dependent on the expression of genes defining BCR signalling has been pivotal, but still lacks in attaining accurate prediction of patient survival in clinical course.

Increased aggregation of B lymphocytes due to a lack of apoptotic signalling has been a well known cause in B-CLL disease pathogenesis, suggesting that B-CLL cells lack the fine tuning of a balance between pro-apoptotic and anti apoptotic factors in the microenvironment. The anti-apoptotic protein Bcl-2 is highly expressed in B-CLL patients and has been well characterized, but paradoxically, high levels of pro-apoptotic molecules have also been observed [30, 31]. Deregulation of the cell cycle regulators (CCND3, CCND1, and MYC) and apoptosis regulators (NPAT, CUL5 and PPP2R1B), and a role for these genes, has been implicated in the pathogenesis of B-CLL disease [32]. Moreover there is a rapidly growing number of the modulators of BCR components and the cell death machinery. These can

be targeted to disrupt the turgid balance between pro apoptotic and anti apoptotic factors, and restore healthy balance, and are considered to be of optimal therapeutic value in the treatment of CLL [33, 34].

4. Microenvironment: Cross talk and interplay

Microenvironment regulating signals are crucial for the process of signaling in leukemic cells that induce proliferation and lead to the survival and accumulation of leukemic cells within lymphoid organs. The *in vivo* accumulation of leukaemic lymphocytes is potentiated by interactions of CLL cells with other cells such as mesenchymal stromal cells (MSCs), nurse-like cells (NLCs), T cells and other soluble factors that include Interleukins (IL-4) and cytokines such as CCL22 and CCL17 [35]. CLL cells grow in the microenvironment of a solid tissue where they initiate as monoclonal disease of autoreactive B cells, due to receptor-ligand interactions resembling an autoantigen and neoantigen. A process called auto antigen drive has been shown to be essential in selecting susceptible B cells out of the normal clone and driving them into the disease state [36].

Common genetic aberrations that occur in CLL disease and different stimuli originating from the microenvironment, cooperate in the selection and expansion of the malignant clone. CLL cells relentlessly accumulate *in vivo* but rapidly undergo spontaneous apoptosis *in vitro*. This implies that their apoptosis resistance, rather than being an intrinsic feature of leukemic stage, depends on external signals for survival since the maturation stages of B cells are highly dependent on microenvironment signals [35].

Exemplified crosstalk between CLL cells and accessory cells occurs in the marrow and/or lymphoid tissue microenvironments. Contact between CLL cells and NLCs or MSCs is established and maintained by chemokine receptors and adhesion molecules. NLCs express the chemokines CXCL12 and CXCL13, whereas MSCs predominantly express CXCL12. NLCs and MSCs attract CLL cells via the G protein coupled chemokine receptors CXCR4 and CXCR5, which are expressed at high levels on CLL cells [35]. CD38 expression allows CLL cells to interact with CD31, the ligand for CD38, expressed by stromal and NLCs. Ligation of CD38 activates ZAP-70 and downstream survival pathways. Self and/or environmental antigens (Ag) are considered a key factor in the stimulation and expansion of the CLL clone. BCR stimulation and co-culture with NLCs also induce CLL cells to secrete high levels potent T-cell attracting chemokines such as CCL3 and CCL4.

Through this mechanism, CLL cells can actively recruit T cells for cognate T-cell interactions with CLL

cells. CD40L T-cells are preferentially found in CLL proliferation centers and can interact with CLL cells via CD40. Cytokines secreted by T-cells or CLL cells such as TNF- α or IL-4 are considered important regulators of CLL cell survival. Moreover, downregulation of proximal and intermediate T-cell receptor signalling cascades and globally reduced cytokine secretion, contributes to significant immunodeficiency in non-leukemic indolent B-cell lymphomas [37]. Collectively, this crosstalk between CLL cells and accessory cells results in activation of survival and drug resistance pathways [36]. Focusing on the therapeutic aspect of regulating the microenvironment, this includes disruption of targets in the complex interplay between CXCR's and adhesion molecules, which is the precise definition of systemic T-cell dysfunction. This might eventually provide a rationale for appropriately tailored molecular therapies targeting not only B-CLL cells but also the microenvironment, to ensure improved treatment.

5. Micro RNAs

Micro RNA's (miRNAs/miR's) are a recently discovered class of molecules that regulate gene expression at the posttranscriptional level. The interactions between miRNAs, target genes, and pathways in CLL are clearly complex, as are the links between genotype and phenotype miRNA machinery, which include miRNAs involved in many cellular processes such as proliferation and apoptosis. Some microRNAs are referred to as oncomiR's and exhibit differential expression level in cancer disease by acting as either oncogenes or tumour suppressor genes [38, 39]. Recent *in vivo* studies demonstrated that tumours became addicted to oncomiR's. Their inactivation resulted in complete tumour regression, demonstrating the importance of miRNAs in therapeutic pharmacological intervention (for example miR-21). A role for miR-21 in cancer has been implicated strongly by the fact that it is overexpressed in haematological malignancies, especially B-CLL [38]. Several studies have proposed the use of microRNA-based classifications as signature profiles for B-CLL disease prediction and survival [41]. miR's studies are likely to have an increasing influence in the diagnosis, prognosis and treatment of human cancers, including CLL [42].

Cimmino et al. [43] proposed that miR-15a and miR-16-1 function as tumor suppressor genes by modulating Bcl-2. The same miR signature was also associated with the presence or absence of disease progression, since Bcl-2 mRNA stability is not affected by the overexpression of these miR's but instead was regulated by Bcl-2 expression at a posttranscriptional level. miR-34a expression has been found to be very low in B-CLL patients. B-CLL cells with reduced miR-34a expression showed increased

viability after DNA damage, independently of 17p status. Low expression of miR-34a in CLL is therefore associated with p53 inactivation as well as chemotherapy refractory disease, impaired DNA damage response, and apoptosis resistance irrespective of 17p deletion/p53 mutation [44]. This study identified the role of miR-34a in B-CLL pathogenesis where they speculated that it took over the control of apoptotic machinery in both p53 dependent and independent pathway by controlling the expression of Bcl-2 and p21 [45].

The demonstration of down-regulation of cyclin-E2, CDK6, E2F5, cyclin D1 (CCND1) and Bcl-2 protein levels after induction of miR-34a expression has been noted to be important, suggesting that miR-34a in CLL mediates its functions within and potentially outside the p53 pathway [46]. A recent study report by Asslaber et al. [47] showed that a low level of miR-34a expression profile predicted a shorter time for treatment of disease in B-CLL patients, alternatively an over expression of miR34-a induces apoptosis through p53 attenuation by negative feedback loop regulatory mechanism. This study specifically indicates that the p53 tumour-suppressor pathways and associated miR-34a expression may be a promising avenue of research into the pathogenesis and prognostic assessment of CLL, and may prove more useful in aiding early-stage identification of patients with fludarabine refractory CLL. Moreover karyotype specific signature profiles have been found to be associated with different forms of B-CLL such as indolent and aggressive [41].

Analysis of miRNA expression values between the predefined subgroups showed that the miR-223, the miR-29b, the miR-29c, and the miR-181 family are down regulated in 17p-aggressive cases showing the highest level of Tcl-1 expression compared with 17p-indolent cases [48]. The findings in this study suggest that interactions between Tcl-1 and miRs play an important role and suggest them as new markers to assess the disease course in CLL patients. Although such findings are exciting, larger studies demonstrating reproducibility are still required. Targeted miRNA therapeutics remain at the early stages of development and are limited primarily to *in vitro* and murine models of disease. Given the fact that miRNA's expression profiles may prove to be novel surrogate markers for common cytogenetic lesions, the prognostic indicators like karyotype specific signature in B-CLL disease is highly difficult to achieve through miRNA based therapy. To conclude, micro RNA studies indicate the importance of considering more than one parameter to assess the tumour burden in B-CLL, but could not advocate a replacement of standard clinical and molecular markers. The research findings suggest that the addition of the miR's expression is critical for the evaluation and more accurate management of CLL patient care.

6. Drug targets and resistance

Adequate progress has been made in recent times for therapeutic intervention and management B-CLL disease. New insights into the molecular pathology of B-CLL have generated a plethora of biological markers that predict the prognosis and influence therapeutic decisions. These markers include historical Rai and Binet staging systems, loss of p53 and ATM functions, the unmutated IgVH gene, and high expression of ZAP-70 or CD38 [49]. These factors in B-CLL cells have been identified as unfavourable prognosis to standard chemotherapy or are indicative of aggressive disease. Several strategies that induce apoptosis and circumvent resistance by a combination of inhibitors of transcription and translation such as CDK inhibitors, together with other approaches that interfere with the function of anti-apoptotic proteins that initiate synergistic killing in B-CLL (flavopiridol, R-Roscovitin, Danciclib and SNS-032) have been employed [50-53].

A recent review by García-Escobar et al. [54] stated that preclinical evidence for the efficacy of signal transduction inhibitors such as alkylating agents [55] and purine analogues [56] in B-CLL is particularly encouraging, but the results of clinical trials on using these as single agents have not been satisfactory. Combinational therapy on the other hand has been proved efficacious and is currently being explored in pre-clinical studies and early-phase clinical trials. These have not been used routinely in the clinic because they exhibit differential responses due to poor diagnostic and prognostic marker relevance. To date drugs like fludarabine, bendamustine and the two monoclonal antibodies, alemtuzumab and rituximab have been approved by European and/or American regulatory agencies. Monoclonal antibody Alemtuzumab holds a promising combination of therapeutic modality with fludarabine as a first line treatment for refractory CLL patients. Recently haematopoietic stem cell transplantation such as low toxic non-myeloablative allogeneic transplant has opened new perspectives in the management of CLL patients, and appears to be a promising therapy for elderly patients.

Additional monoclonal antibodies targeting CD20, CD23, CD37, CD38 or CD40, as well as drugs designed to interfere with proteins regulating the cell cycle, apoptotic machinery or leukemic microenvironment (e.g., flavopiridol, oblimersen, ABT-263 or lenalidomide) are being investigated in clinical trials. To date, single-agent clinical trials have indicated that the major clinical outcome is the stabilization of disease states. Lyse [57] showed that pre-treatment of TRAIL resistant B-CLL cells with histone deacetylase inhibitors such as Oxam, rendered cells to be susceptible for subsequent killing by recombinant TRAIL protein. This therapy preferentially

increased surface expression of TRAIL-R1 and TRAIL-R2, there by leading to caspase 8 and caspase 3 activation. Importantly, this is a combination therapy that enables preferentially sensitized transformed cells to apoptosis. Recently Niedermeier et al. [58] and Herman et al [59] demonstrated that the isoform-selective PI3K inhibitors induced B-CLL cell apoptosis *in vitro* in MSC co-cultures synergized with fludarabine induce B-CLL cell apoptosis. Thus, combination therapies using specific and recombinant inhibitors in signal transduction pathway might prove to be efficacious for the treatment of B-CLL.

The main focus of therapeutic strategies in B-CLL is still cytotoxic chemotherapeutically available alkylating agents or purine analogues that trigger DNA damage response via p53 leading to a prominent cell death. Most of B-CLL diseased patients carry defects in the p53 pathway and therefore it has been challenging to overcome the resistance through p53 independent cell death pathways [60, 61]. Drug- induced resistance to B-CLL treatment enhances by activating survival signaling pathways (for example NF- κ B) through activation of PI3K-Akt and therefore counteracting the effect of the treatment. Microenvironment plays major roles in B-CLL tumour resistance to therapies. Most of the factors and pathways discussed above that are responsible for sustained activation of survival pathways in B-CLL are provided by contact with the microenvironment.

7. Systems biology approach

Although clinical stages such as Rai and Binet tests are the required basis for the prognosis in the B-CLL patients, many other biological markers mentioned in the above section have been offering important insights to prognostic information. Nonetheless these prognostic factors have not been fully validated or standardized in large clinical trials. Moreover, it is practically impossible to validate all possible biomarkers in clinical trials due to the fact that response upon drug dosage in B-CLL patients is varied, and therefore could not sustain in clinical evaluation. Existence of conflicting data in research studies has added more complexity and confusion in the evaluation of B-CLL disease pathogenesis. For example Klein et al [22] reported that a large number of apoptotic genes deregulated in particular with 17p deletion, which reflected the distinctly aggressive biology of a subgroup cohort study in B-CLL patients and contrasted with reports published by Stankovic et al [62] and Schaffner et al [63], showing an indistinguishable native gene expression pattern in terms of quantitative folds when comparing wild type and TP53-mutant in B-CLL cells acquired from patients.

Notably, Klein et al [22] used highly sensitive quantitative RTPCR approach and a greater number of

case studies in evaluating the data, when compared to the Stankovic et al. [64] microarray study. Moreover, low Bcl-2 levels in 17pdel, the subgroup with the worst clinical course was reported by Klein et al [22] due to the known anti-apoptotic function of Bcl-2 and the association of high Bcl-2/BAX ratios with aggressive disease, which argues against a relevant role of Bcl-2 overexpression or elevated Bcl-2/BAX ratios in the pathogenesis of the B-CLL or in B-CLL prognosis. The Kim et al. [65] study supported the use of a whole genome sequencing approach for comprehensively decoding the B-CLL genome in order to better understand the genetic defects caused by insertion, deletion, base change, and restriction site polymorphism present in both coding and non-coding regions in hundreds of loci within the genomes, which indicate the wide presence of small genomic aberrations in B-CLL. B-CLL cases display recurrent genetic aberrations including trisomy 12 and monoallelic or biallelic deletion/inactivation of chromosomal regions 17p, 11q and 13q14 [66]. Presently these deletions and the mutational status of the IgVH gene provide prognostic information and may determine the type of therapy [67].

The above mentioned molecular signatures may have a profound impact on prognosis for B-CLL disease pathogenesis, but the lack of routine clinical methods for diagnosis is a major challenge which should be accounted for in future research. Taking into consideration our current understanding of B-CLL disease pathogenesis, it is now convincing that application of systems biology should be employed as an approach for predicting patient response and designing individualized therapies. An iterative interplay between biological experiments producing quantitative data followed by suitable modeling strategies should enable us to interpret the biology in a global system perspective, thereby increasing our current understanding of the signaling pathways involved in the pathogenesis of a complex disease such as B-CLL. This approach should also include the extracellular aspects of the disease mechanism such as microenvironment. Due to the fact of these differential study conclusions, it is now time to consider prognosis and treatment on an individual basis rather than cohort studies involving large numbers of patients. Moreover it is now evident that B-CLL is not a single target regulated disease, but rather has multiple targets with two distinct disease phenomenae (indolent and aggressive forms).

Building network models by applying systems biology is an approach that will enhance our understanding of key molecular players in the B-CLL disease pathogenesis. To generate a computational model for prognosis of B-CLL patients *in silico*, one has to overcome a number of challenges such as working with a large number of chemically diverse molecules that are often rapidly changing their concentrations in terms of their

expression and regulation [68]. In order to overcome these challenges, various sensitive detection systems or gene reporter systems should be implemented to detect the optimal expression *in vivo* [69] before its execution in building a model. It is clear from the many experiments and studies carried out to date that there are no single genetic or molecular aberrations which alone lead to the B-CLL phenotype.

Disease progression can vary considerably from one patient to another suggesting that future therapies will need to be designed on an individual basis. To do this, it requires an in-depth analysis of two major groups of variables. Firstly by encompassing the molecular interactions between the various intra-cellular proteins which implement the cell's regulatory pathways, in particular survival, anti-growth, proliferation and apoptosis (Figure 1). Secondly, as these interactions are highly complex at the intra-cellular level where the loss of homeostasis has to be analysed, a model reporting on the molecular activity, and the imbalance between B-cell proliferation and apoptosis should be addressed. Due to this complexity, some form of holistic approach to the problem is required and we believe that one way forward is to describe the dynamics of the various molecular and cellular interactions by using a mathematical model. In this way the mechanistic links, which underpin the B-CLL disease phenotype can be defined and, subject to calibration at the individual patient level, these models would enable a better understanding of disease progression, as well as providing a methodology for targeted drug interventions.

The key aspects in cellular signaling that involve the cell cycle, apoptosis and various intracellular pathways such as DNA damage repair and cytokine receptor activated network activating PI3K/AKT, are considered to be crucial components that are frequently deregulated in B-CLL disease. Each pathway is subject to an intensive study by the computational systems biology approach: modelling of the cell cycle [65-73], apoptosis [74-76], MAPK and PI3K/AKT pathways [77, 78]. As these pathways are involved in cell proliferation, survival and cell death, computational models of these pathways are considered promising tools in cancer research for prediction of cancer disease progression, development of biomarkers, and drug therapy efficacy [79-82]. Henceforth, understanding the complex regulation of B-CLL disease pathogenesis, requires a strategic knowledge on the models of the cell cycle, and apoptosis that report on intrinsic and extrinsic cell signaling network. Systems computational approach to the complex regulation in B-CLL pathogenesis is now at the initial stage of development. We discuss below recent results from computational modeling of these pathways and address some specific results relevant to B-CLL disease.

The mathematical approach to modelling the cell

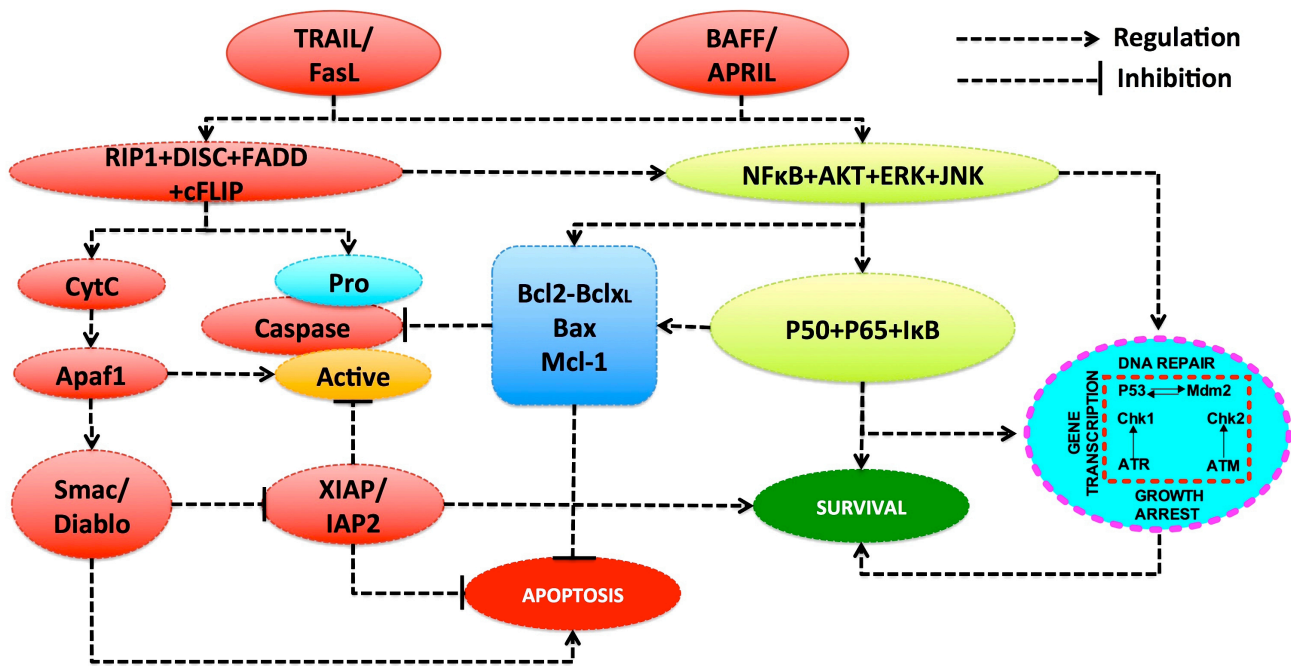


Figure 1. An extract of the intra-cellular network, frequently deregulated in B-CLL disease. The diagram shows the interactions upon ligand stimulation of TRAIL, FaS, BAFF and APRIL receptors and the activation of key proteins that are involved in regulating B-cell apoptosis or survival by regulating gene transcription. It illustrates the function of Mdm2 and P53 as activators of various cell cycle checkpoints regulating proteins such as CHK2, CHK1 and DNA damage repair enzyme ATM and ATR. Regulation of this network can lead to growth arrest and DNA repair in promoting cell survival. Any aberration in these parameters induced by drug will induce caspase activation by its upstream target Bcl2/Bax and Mcl-1. The construction of mechanistic models of the underlying dynamics could enable better targeted drug therapy.

cycle was developed in a series of works by Novak and Tyson [70, 83, 84], which demonstrated that the cell cycle could be modelled as an irreversible sequence of changes between successive steady states, driven through changes in cyclin levels and loss of their stability at cell cycle phase transition. Further development of this approach was made in a series of models which successively described key features of cell cycle dynamics, and predicted main control mechanisms of the transitions in the cell cycle (see a review of cell cycle models in [85]). The general result of cell cycle modelling showed the extreme robustness of cell cycle function at a wide variation of model parameters such as protein-protein interaction constants, rate constants of protein phosphorylations, protein expression and degradation rate constants [70, 80, 81]. These findings are in accordance with experimental observation that the cell cycle is one of the highly controlled processes in the cell [84]. The decision making for cell division, its delay, arrest or apoptosis is tightly orchestrated by integrating both external cellular signals, such as growth factors and hormones, and internal signaling molecules. Cell cycle modelling demonstrated that despite extensive control mechanisms in regulating phase transitions, it can lose its control and become a fragile system when variations in specific parameters of the model that affect robustness are introduced. From a clinical point of view, this fragility in

the system can be referred to as cell cycle malfunction, observed as uncontrolled cell proliferation, a common phenomenon underlying many cancers [82].

7.1 Identification of fragile nodes

In this context a new area in systems biology is being developed that aims at not only the description of the normal functioning of cellular control systems, but also the modelling of the mechanics of their malfunction in pathological conditions including cancer [74, 81, 86]. Novel approaches were designed to analyze the existing models of the cell cycle to study the abnormality in its control mechanisms leading to uncontrolled cell proliferation and survival, causing diseases including solid and hematological cancers [80, 82]. One of the challenges in systems biology is in determining the fragile, sensitive nodes of signaling pathways, including the cell cycle regulation, which cause the disruption of the balance between cell proliferation and death. These fragile points are suggested as control hubs within cellular signaling networks, which are extremely sensitive to mutations or the aberrant protein expression observed in cancers [82]. These fragile hubs, possessing increased sensitivity to internal perturbations, are assumed to also be sensitive to external perturbations such as drug action. Fragile points in signaling networks therefore represent

promising targets for drug intervention [80-82].

One of the powerful methods for determining fragile nodes within cellular signaling networks is sensitivity analysis of the computational models [79, 82]. Sensitivity analysis of the model consists of the calculation of sensitivity coefficients: 'S' which represents the response of a network output, 'ΔY' to a variation of model parameters, 'Δp' (protein-protein interaction constants, rate constants of protein phosphorylation, protein expression, degradation rate constants, others): $S = \Delta Y / \Delta p$. Sensitivity analysis permits ranking sensitivity of different outputs of the model to variations of the model parameters and determining a network locus which is most sensitive to these perturbations. Points in the network that exhibit extreme sensitivity to small perturbations of the parameters are often referred to as "fragile" (the converse of "robust") [74, 80].

The results of sensitivity analysis of the cell cycle models obtained by Nayak S et al. [82], screened for fragile mechanisms in the existing cell cycle models using sensitivity analysis and predicted that such mechanisms are implicated in various solid and hematological cancers including B-CLL. The sensitivity coefficients calculated in sensitivity analysis [82] of the G1/S model of Qu et al. [71] and the G2/M-DNA damage model of Aguda

[72] are shown in figure 2. Sensitivity coefficients with high ranks showed that the following reactions can be classified as the most fragile steps in the G1/S checkpoint: 1) dephosphorylation of CDC25, 2) expression of cyclin E, 3) degradation of the cyclin E-CDK2 complex, and 4) level of the transcription factor E2F. Experimental and clinical data supported the computational identification of the fragile reactions in the cell cycle which represent promising targets for drug therapy. The first ranked reaction is the activation of CycE-Cdk2 by CDC25 phosphatases in the G1/S network. The significant role of CDC25 phosphatase was observed in many solid and haematological cancer progressions, and several CDC25 inhibitors showed promising results in cancer treatment [82].

The second ranked fragile mechanism in the G1/S network obtained in sensitivity analysis is cyclin E-CDK2 complex. Inhibition of the active cyclin E-CDK2 is being considered as a treatment strategy in different types of cancer including B-CLL. For example, a synthetic flavone, flavopiridol induces cell cycle arrest by inhibiting multiple CDKs, and leads to p53-independent apoptosis in CLL cells [87]. The first ranked fragile points in the G2/M-DNA damage network is the activation of preMPF (cyclin B-CDK1 complex) catalyzed by CDC25 [82]. This

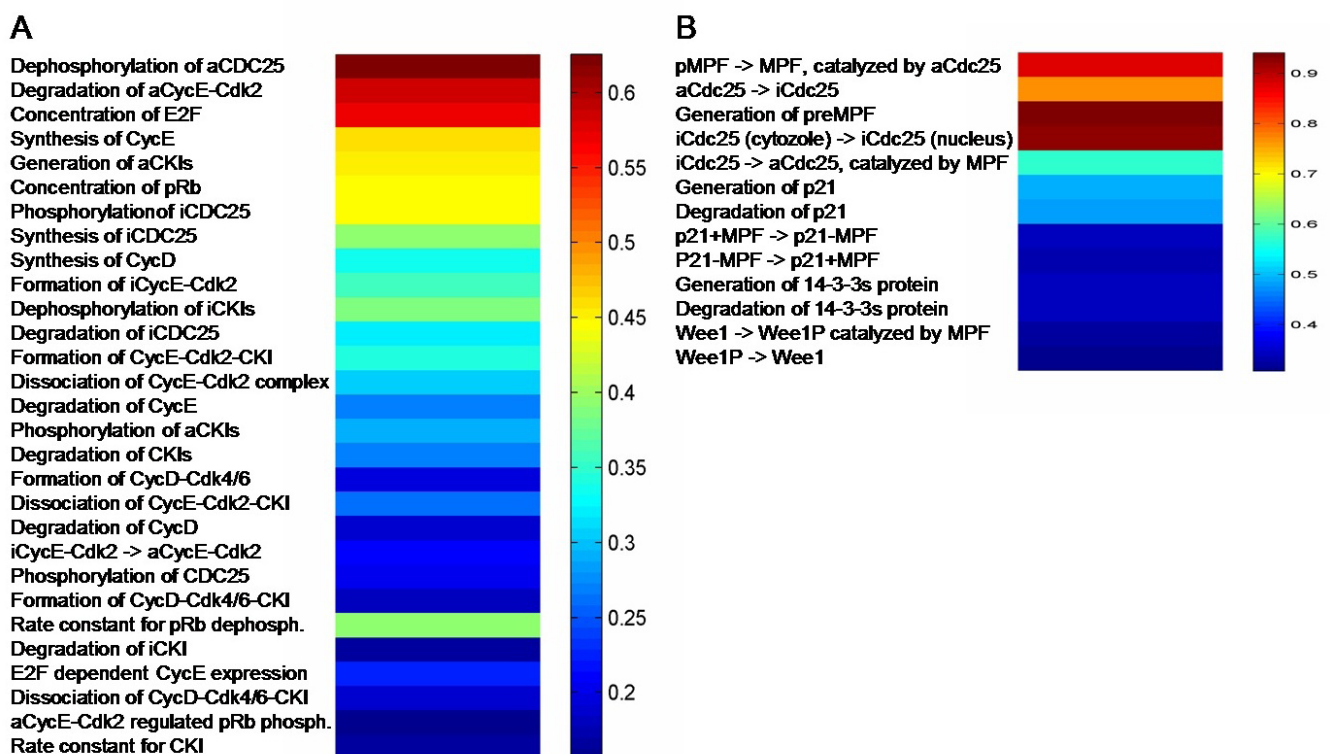


Figure 2. Sensitivity analysis of cell cycle models. Sensitivity coefficients [82] determined for the G1/S model [66] (A) and the G2-DNA damage model [67] (B). Abbreviations: CycE - cyclin E, aCycE/iCycE – active/inactive CycE, CDC25 - a dual-specificity phosphatase CDC25A, aCDC25/iCDC25 – active/inactive CDC25, Cdk2 - cyclin dependent kinase 2, E2F – transcription factor E2F, CKI - a cyclin dependent kinase inhibitor, aCKI/iCKI – active/inactive CKI, pRb - retinoblastoma protein, CycD - cyclin D, Cdk4/6 – cyclin dependent kinase 4 or 6, pMPF - pre-maturation promoting factor, p21 - cyclin-dependent kinase inhibitor, 14-3-3s - 14-3-3s protein, Wee1 - Wee1 kinase. Data are represented by courtesy permission of Prof. J.D. Varner.

finding correlates with the CLL treatment by bryostatin-1, a protein kinase C (PKC) inhibitor and antagonist of cyclin B-CDK1 complex [88]. The other types of fragile mechanisms obtained in sensitivity analysis [82] are related to protein synthesis and degradation (see sensitivity coefficients for concentrations of E2F, synthesis of CycE, CDC25, and other proteins in figure 2). Targeting these mechanisms now becomes an active area of therapeutic strategy by developing the manipulation methods of translational machinery and programmed proteolysis [82].

7.2 Signalling simulation models

The signaling pathway relevant to B-CLL disease is PI3K/AKT pathway, which plays a key role in cell proliferation, differentiation and survival [58, 59]. Constitutive activation of this pathway by chemokine and cytokine receptors in CLL cells results from interaction of leukaemia cells with their environment, mainly due to chemokines secreted by stromal cells, which activate CXCR4 receptors and downstream pro-survival PI3K/AKT signaling [89]. Computational modeling of the PI3K/AKT pathway was a subject of many theoretical studies, which focused on different aspects of the complexity in this signaling pathway [77, 78, 90-92]. Development of large-scale models of the PI3K/AKT network, reported the response of the signaling network upon different cytokine stimuli [91] and elucidated the control mechanisms in AKT signaling cross talk with other pathways [92]. The application of the computational modeling of PI3K/AKT signaling in cancer research [93] is aimed at the description of activation signaling in different cancer cell lines [78],

analysis of the roles of cancer-driven mutations in this pathway [78,94], modeling of the response of the pro-apoptotic signal to anticancer drugs [78, 92], elucidation of mechanisms of drug resistance [95], identification of drug targets, [78, 96], optimization of anticancer therapy, and also the development of combination therapy [78, 97].

Sensitivity analysis of these models being a powerful method for analysing PI3K/AKT signaling in cancer was carried out in many works to identify fragile points in this pathway [78, 91, 96, 97], to determine sensitivity of the pathway to different mutations involved [97], and to find promising drug targets [78, 96, 97]. Note the different methods of sensitivity analysis used in different works [78, 91, 96, 97]. Below we discuss the results of sensitivity analysis of PI3K/AKT pathway model carried in [96, 97], which can be used to analyse drug targets within this network.

The results of sensitivity analysis of the PI3K/AKT pathway model [97] are given in figure 3. The comparative analysis of sensitivity coefficient ranking for different network modules revealed that one of the most sensitive loci within this network is the PI3K/PTEN cycle which forms a regulatory hub within cellular signaling. It includes PI3K and phosphatase PTEN, which jointly control the pool of the second lipid messenger, phosphatidylinositol-3,4,5-trisphosphate (PIP3). This conclusion is in agreement with the results of an integrative genomic and proteomic analysis of solid [98] and haematological cancers [99] which revealed that this regulation hub is vulnerable to mutations in cancer. PI3K is considered an

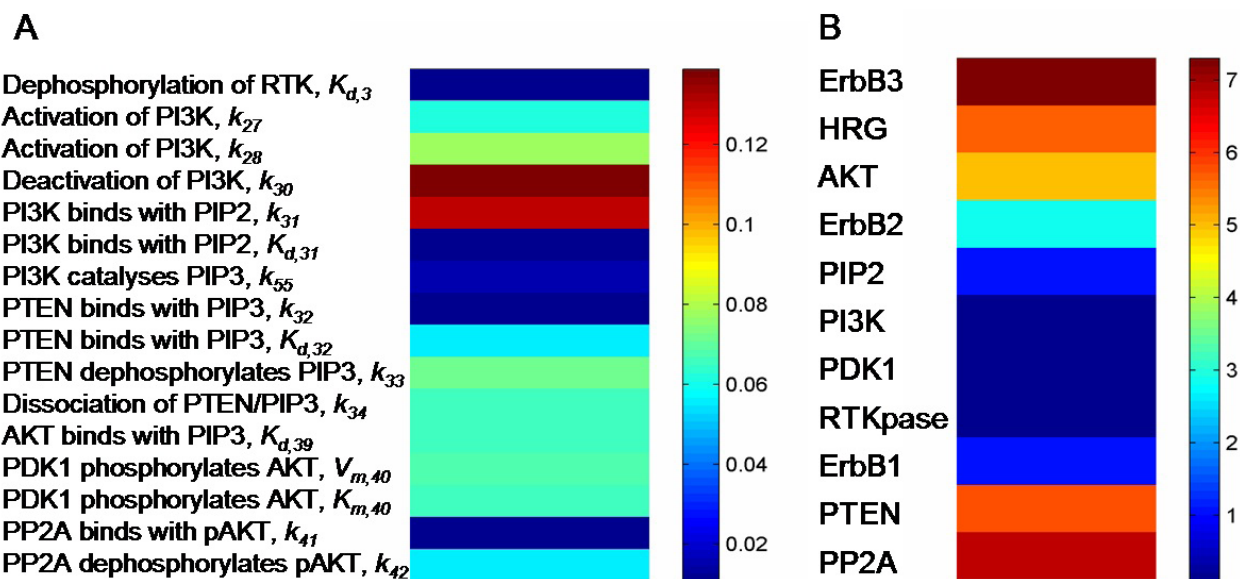


Figure 3. Sensitivity analysis of the PI3K/AKT signaling model [91]. Representation of sensitivity of phosho-AKT signal to kinetic parameters: rate constant k_p , dissociation constants $K_{d,i}$, maximal rate of reaction $V_{m,i}$ and Michaelis-Menten constants, $K_{m,i}$ (A) and to the initial concentrations of the enzymes (B). Abbreviations: AKT - protein kinase B, pAKT - phosphorylated AKT, PI3K - phosphatidylinositol-3-kinase, PTEN - phosphatase and tensin homolog, PDK1 - phosphoinositide-dependent protein kinase-1, PP2A - protein phosphatase 2A, PIP3 - phosphatidylinositol (3,4,5)-trisphosphate, PIP2 - phosphatidylinositol 4,5-bisphosphate.

attractive treatment for targeting several types of cancers including B-CLL [89]. The recent development of specific PI3K inhibitors targeting different PI3K catalytic subunits enable the inhibition of the p110 δ isoform of PI3K, which is reported to be specific to hematopoietic cells [58, 89]. Clinical testing of CAL-101, a selective inhibitor of the PI3K p110 δ isoform showed a suppression effect on stromal cell-derived survival of CLL cells [89, 100].

7.3 Apoptosis simulation models

The high sensitivity of PI3K/AKT signalling to AKT expression level revealed in sensitivity analysis (see figure 3B) showed its significant impact to anti-apoptotic signal. Activated AKT inhibits apoptosis by phosphorylation of Bad protein, procaspase-9, and involves in NF- κ B activation, so AKT inhibition is an attractive target for CLL drug therapy. Preclinical trials showed that AKT inhibitor, A-443654 induces apoptosis in B-CLL cells [101, 102]. The cell cycle and PI3K/AKT pathways discussed above are directly connected to the apoptotic signalling pathway which plays a significant role in the pathogenesis in B-CLL, and is considered an attractive target in the development of novel strategies in CLL therapy [89].

A large number of theoretical studies are devoted to computational modelling of both the intrinsic and extrinsic apoptotic pathways [74, 75, 103-110]. The theoretical modeling of intrinsic apoptosis pathways are focused in part on apoptosis induced by DNA damage signals, and includes the description of p53-Mdm2 pathway with ATM/CHK2, ATR/CHK1 and p21 components, which regulate DNA repair, cell cycle arrest, and apoptosis (see figure 1) [80, 104, 106, 108]. Sensitivity analysis of p53-Mdm2 pathway models showed that the dynamics of the DNA damage response are sensitive in part to the level of p53 which dynamically changes in different cells [106, 108]. The fragility of the DNA damage pathway leading to promoted cell survival and genome instability, can play a particularly significant role in pathogenesis of B-CLL [89]. TP53 mutations are considered to be biomarkers of the overall survival, treatment-free survival, and relapse in CLL patients who showed the high prognostic value independent from del[17p]. 4.5% of untreated patients with CLL have TP53 mutations in the absence of del[17p]. Mutations in TP53 are detected in about 10%-15% of patients with CLL at diagnosis, and 30 % on combination with del[17p] [83]. p53 deregulation alone or concomitant with del[17p] is reported to be a significant factor in the effective treatment option for these patients [89]. Additionally, the ATM components of the DNA damage pathway was observed to be deregulated in B-CLL cells and might underlie refractory disease [63, 89].

The extrinsic apoptosis is triggered by binding the tumour necrosis factor receptors (TNF-Rs) with members

of TNF family ligands (TNF- α , FasL, and TRAIL) [74]. Computational modeling of the extrinsic apoptotic pathway revealed key regulators of TNF-mediated apoptosis. Particularly important role plays the positive feedback in the bistability dynamics of apoptotic signals (i.e. all-or-none behaviour) and irreversibility in caspase activation, as well as multi-factorial controlling type I versus II apoptosis pathways [74, 75, 105, 106, 109-112]. Sensitivity analysis of TNF-mediated apoptosis models aimed at identifying control points in apoptotic signalling [75, 103, 107, 109-111, 113]. The results of sensitivity analysis of the computational models of apoptosis induced by TRAIL [114] and FasL [75] are given in figure 4A and B, respectively.

The high level of sensitivity of the model output (caspase-3 activation) showed the following sensitive points of this pathway: 1. kinetic parameters of TRAIL ligand-TRAIL receptor interaction and concentrations of membrane receptors; 2. activation of pro-apoptotic protein Bak/Bax negatively regulated by Bcl-2 protein; and 3. Smac/XIAP inhibition of caspase-3 and caspase-9 resulting in positive feedback in apoptosis. The sensitivity of caspase-3 activation to Bcl-2 concentration was obtained at upregulation of Bcl-2 as opposed to insensitivity to Bcl-2 at downregulation of Bcl-2 when Bcl-2 does not inhibit the type II apoptotic pathway significantly (see the representation of the sensitivity coefficients at the decrease and increase of protein expression levels in figure 4B) [75]. This finding is in agreement with clinical data on the significant role of Bcl-2 in B-CLL [89]. The B-CLL cells, acquire survival benefit against apoptotic signals, and resistance to therapy by upregulation of Bcl-2 [87]. Overexpression of Bcl-2 is considered as one of the biomarkers of CLL and a promising therapeutic target in CLL therapy as alone and in combination. Inhibition of Bcl-2 protein family activates programmed cell death through initiation of the mitochondrial pathway of apoptosis. Now several therapeutic agents targeting Bcl-2 family of proteins are in clinical development. Oblimersen (Bcl-2 antisense oligonucleotide) is at phase III trial and small-molecular mimetics of the BH3 peptide domain of Bcl-2 (obatoclax, navitoclax) are being clinically evaluated in patients with repulsed/refractory CLL [89, 115]. The different sensitivity of mitochondrial apoptotic pathway towards down-regulation and up-regulation of Bcl-2 revealed in sensitivity analysis showed that Bcl-2 inhibitors can possess selectivity for CLL cells versus healthy cells with a normal expression of Bcl-2 [75].

High sensitivity of caspase-3 activation with the affinity of TRAIL ligand to TRAIL receptor and their concentrations obtained in sensitivity analysis (figure 4), correlates with a significant role of the death receptor in initiation of apoptosis in B-CLL cells [101]. It was observed that CLL cells manifest resistance to TRAIL-

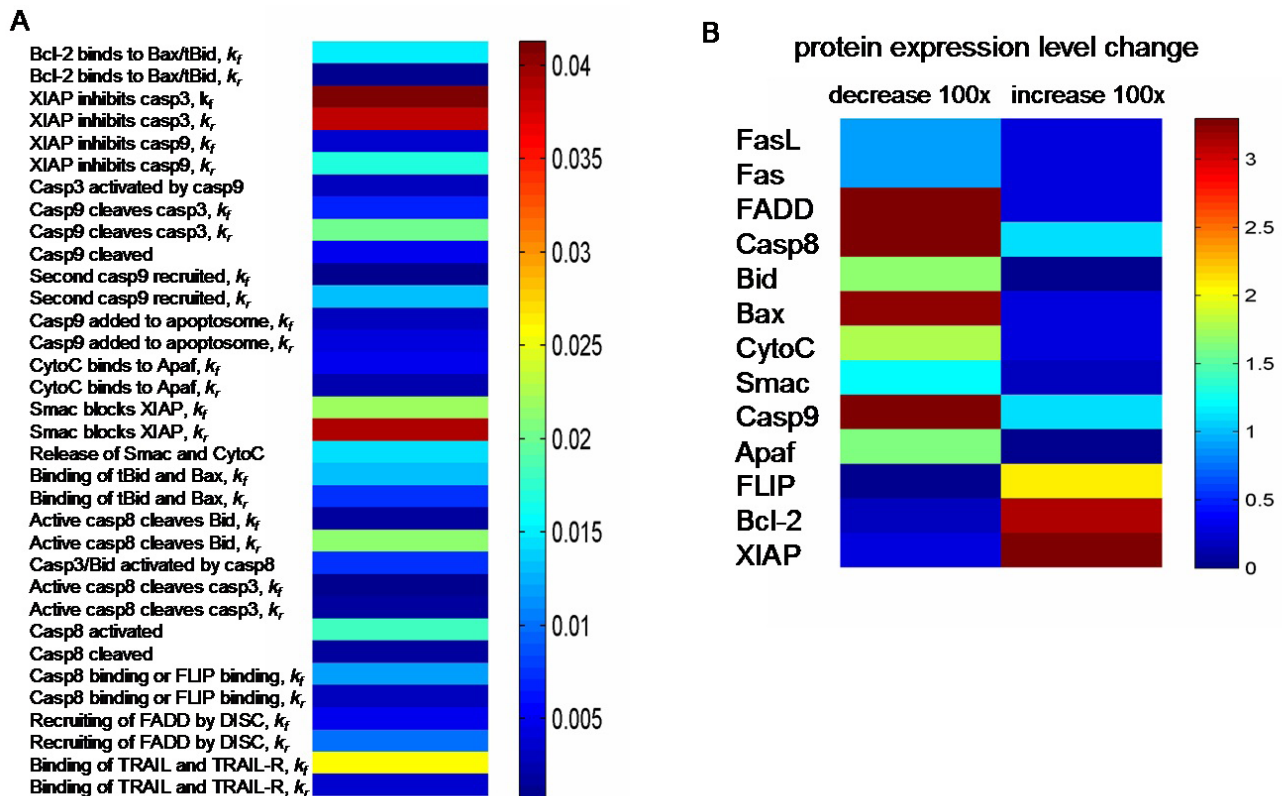


Figure 4. Sensitivity analysis of the apoptosis models. (A) Results of sensitivity analysis of the model of TRAIL-induced apoptosis to kinetic parameters of the model: the rates of the forward, k_f and reverse reactions, k_r [108]. (B) Results of sensitivity analysis of the model of FasL-induced apoptosis to concentration of the proteins involved [69]. Data represent the change in the half-time of caspase-3 activation (hour) at the variation of protein level two orders of magnitude in either direction from the baseline values. Abbreviations: Fas - FAS receptor, FasL - Fas ligand; XIAP - X-linked inhibitor of apoptosis protein, FADD - Fas-associated protein with death domain, casp3 - a caspase-3 protein, casp9 - a caspase-9 protein, casp8 - a caspase-8 protein, cytoC - cytochrome c, FLIP - flice inhibitory protein, Bax - Bcl-2 associated X protein, Bcl-2 - B-cell lymphoma 2 protein, Bid - BH3 interacting domain death agonist, TRAIL- TNF-related apoptosis-inducing ligand, TRAIL-R – TRAIL receptor, Smac - second mitochondria-derived activator of caspase, Apaf-1 - apoptotic protease activating factor-1. Data are represented by kind permission of Prof. Z. Sun (A) and Prof. D.A. Lauffenburger (B).

induced apoptosis due to a low surface expression of death receptors, or over-expression of inhibitory proteins such as FLIP, IAP2, and XIAP [101, 116]. Sensitising B-CLL cells to TRAIL-induced apoptosis is possible by up-regulating surface expression of death receptors or down-regulating the inhibitory proteins [101]. Targeting TRAIL receptors by anti-TRAIL-R1 antibody, mapatumumab also showed *in vitro* efficacy in different haematological malignancies [101]. Apart from pro-apoptotic signaling, the TNF-family receptors activate pro-survival signaling cascades, mainly through the NF- κ B pathway [89, 117].

The decision making in cell survival/apoptosis is determined by the balance between these competing signals. Two ligands that belong to the TNF superfamily, BAFF (B-cell-activating factor), APRIL (a proliferation-inducing ligand), and their receptors, were reported to be key regulators of B-CLL cell survival [89]. Receptors of BAFF and APRIL are expressed on B-CLL cells, and when activated can promote B-CLL cell survival. The release of BAFF and APRIL through an autocrine mechanism

is reported to cause the constitutive activation of both the canonical and noncanonical NF- κ B pathways [117, 118]. Computational modelling of NF- κ B pathway was carried out to elucidate specific mechanisms governing NF- κ B-induced gene expression, as in part-mechanisms of oscillatory dynamics in this pathway [104, 113, 119-121]. Sensitivity analysis of NF- κ B pathway models revealed that formation and function of the inhibitor I κ B kinase complex (IKK), significantly impact on the NF- κ B signalling: [104, 119, 120]. The inhibitors of IKK β that inhibit canonical NF- κ B pathway are expected to be attractive targets for B-CLL therapy [117]. In *in vitro* testing of the action of IKK β inhibitor, UTC on B-CLL cells showed suppression of the protective effects of BAFF on B-CLL cell survival [117]. The other therapeutic approach to inactivate pro-survival NF- κ B pathway in B-CLL cells is developing, and in part designing, a fusion protein targeting both BAFF and APRIL ligands [89, 122]. In addition, novel compounds with pro-apoptotic activity should also be considered as potential for potential treatment of B-CLL patients [123, 124].

8. Concluding remarks and perspectives

Building regulatory network model is based on establishing the dynamics of interaction networks showing regulatory pathways involved in gene regulation during the cell cycle and apoptosis. These network models only form a very small part in terms of calibrating of the models, this can be done using patient samples to produce data in terms of the quantitative measurement of key regulatory proteins and cytokine profiling. Once calibrated on an individual patient basis, the models would then enable monitoring of that patient to determine significant changes in the molecular phenotype and, in terms of therapy, permit intervention therapies to be designed using a broad range of bio-therapeutic drugs. A multi-target approach would be possible by identifying key regulators that overcome drug resistance trigger apoptosis. Moreover by determining sensitivity nodes or fragile points to induce apoptosis in B-CLL cells, can be useful as a robust drug sensitivity reporter model. These models can result in achieving maximum benefit in treatment regimes for B-CLL patients. Due to the inherent complexity however, particularly in regard to the number of regulatory feedback loops, we believe that a successful approach can only be achieved using mathematical methods.

Modelling the cell cycle has been shown to be extremely robust in terms of the parameter variations. Experimental and clinical data support the hypothesis that computationally identified fragile interactions in the cell cycle represent promising targets for drug therapy

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