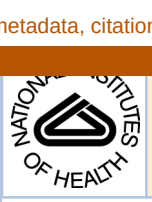

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New experimental and theoretical investigations of hematopoietic stem cells and chronic myeloid leukemia

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Abstract

We report on a focused workshop of The Leukemia and Lymphoma Society that was held at Goldsmiths, University of London in 2008. During this workshop we discussed new clinical and experimental data in chronic myeloid leukemia (CML) research, particularly focusing on the validity (or otherwise) of corresponding mathematical models and simulations. We were specifically interested in whether the models could shed light on any of the fundamental mechanisms underlying this disease. Moreover, we were aiming to form a new community of clinicians and modelers looking at this disease and to define a common language and theoretical framework within which collaboration could flourish.

The workshop showed the role that models can play, not just in trying to fit to existing data or predicting what individual mechanisms or system behaviors might occur, but also in challenging the orthodoxy of the concept of a stem cell and concepts such as "differentiation" and "determination". For years the prevailing view of a stem cell has been an entity (object) with a fixed set of behaviors and with a pre-determined fate. New perspectives in modeling, coupled with the new data that are being accumulated in the genesis of CML and its treatment, questions these assumptions. We propose how we can reach a consensus about a functional view of stem cells in a more continuous and flexible way and how, within this context, we can investigate the significance of modeling results and how they might impact on our interpretation of experimental observations and the development of new clinical strategies.

This paper reports on the workshop and the state-of-the-art models and data from experimental and clinical trials, and sets out a roadmap for more interdisciplinary collaboration between modelers, wet-lab experimentalists, and clinicians interested in CML. It is our strong belief that a more integrated and coherent interdisciplinary approach will further advance the treatment of CML in future years.

Keywords

Mathematical modelling; Computer simulation; Hematopoietic stem cells; Chronic myeloid leukemia

Introduction

During the last decade our increased knowledge of molecular mechanisms underlying the pathogenesis of many types of cancers provides the basis for new therapeutic strategies. One

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prominent example for the successful application of specifically designed compounds that interfere with signaling pathways controlling the survival and proliferation of malignant cells is the treatment of chronic myeloid leukemia (CML) with the tyrosine kinase inhibitors (TKI), imatinib mesylate (imatinib) and second generation agents, dasatinib and nilotinib [1-4]. Such treatments, targeted at the oncoprotein causing the disease, allow for specific inhibition of malignant cells, while largely sparing normal cells. Although the introduction of imatinib as the frontline therapy for CML led to a dramatic improvement in therapy compared to previous treatment options, there are a number of questions that remain. From a clinical perspective the major problems are the persistence of residual disease and the occurrence of TKI resistance [5-8]). Additionally, there are a number of conceptual questions that cannot be answered. For example, although many details of the molecular properties of the TKI molecules are known, the resulting systemic effects – the regulatory responses at the cell population level – still remain unclear. To tackle these problems, mathematical modeling and simulation studies provide powerful complementary approaches (e.g. [9-11]). Such techniques, which are sometimes summarized under the term *systems biology*, can enable a comprehensive understanding of regulatory mechanisms and the resulting treatment effects. Moreover, we are at the stage where we can propose possible *mechanisms* at the cell or tissue level that may result in the systems effects that are observed.

To discuss the potential of mathematical and computational approaches in leukemia research, a Focused Workshop of the Leukemia and Lymphoma Society entitled *Stem Cells and Leukemia — Concepts, Models, Simulations* was held from April 24th – 26th at Goldsmiths College, University of London. In total 24 scientists from Australia, France, Germany, Switzerland, the United States and the United Kingdom attended the three day meeting. It was the central aim of this workshop to bring together theoreticians, experimentalists, and clinicians to discuss the application of theoretical methods for achieving a better understanding of leukemic stem cells (LSCs) and to propose methods to optimize leukemia treatments with the particular focus on CML. In particular, the workshop was intended (1) to communicate the potential and the limitations of theoretical methods in the context of experimental and clinical research, (2) to outline what requirements models need in order to be accepted by clinicians/experimentalists, and (3) to identify clinical and experimental problems that could benefit from the application of mathematical models. An important aspect of the workshop was the participation of scientists who had a clinical background (one-third of the attendees), demonstrating the growing recognition of the value that the *systems biology* approach can bring to clinical research.

In this paper we will report on the major discussions of the workshop outlining where there was consensus and where there was disagreement. As the key goal of this workshop was to bring mathematical and computational modeling more into the mainstream of leukemia research, the final chapter will provide a road map of how we can best bring about a more coherent and focused collective effort in understanding CML.

Towards a consensus view on hematopoietic stem cells (HSCs)

A critical presumption for a successful conceptual discussion on stem cell disorders, such as CML, is an agreement on *what constitutes a stem cell*. In this section we outline our (in the main) consensus view on stem cells but also discuss where there were issues of disagreement or lack of clarity with definitions and concepts.

Identifying stem cells

There is a broad consensus that *functional testing* is the hallmark characterization of tissue stem cells in general and HSCs in particular. Although there are a number of sophisticated

purification and enrichment protocols available [12-15]), there are no techniques as yet to allow for a prospective identification of stem cells at the single cell level. Furthermore, until now there are no validated phenotypic or genomic signatures that can unequivocally characterize HSCs. In other words, the only way to determine whether a particular cell is a HSC or not, is to challenge its cellular function in a specific assay system. The most rigorous of these assays for HSC is the *in vivo* repopulation of irradiated mice using transplantation. In these experiments, if the criteria of *long-term reconstitution and maintenance of functional tissue* (i.e., multipotent differentiation capacity) including the re-establishment and maintenance of a population of cells with (secondary) repopulating potential (i.e., self-renewal capacity) have been fulfilled, then we have demonstrated that at least one HSC was contained in the transplanted cells. Even more, if only one individual cell had been transplanted with the same result, one could then conclude with certainty that this particular transplanted cell was a HSC.

Uncertainty of stem cell characterization

There are some unavoidable problems with a functional definition. First, the assay gives only a definite result if positive stem cell activity is observed. In the situation where a negative assay results (i.e., where there is no long-term reconstitution), the question as to whether the transplanted cells did not have stem cell potential or whether they simply did not use it for whatever reason, cannot be answered. Secondly, even when what we see is a positive repopulation assay and can demonstrate that HSCs *must have existed* in our original population, it does not allow for a *prospective identification* of those cells. The original cells have to *actively* respond to the assay system in order to demonstrate their stem cell potential and, therefore, ultimately change their characteristics. That means, the test systems itself changes the object to be tested. Although not identical, this type of *uncertainty* has something in common with Heisenberg's Uncertainty Principle in quantum physics [16], an analogy that was introduced into stem cell biology almost two decades ago [17]. This type of system intrinsic uncertainty is not the same as statistical uncertainty; it cannot be eliminated by increasing sample sizes of experiments. However, even though a prospective characterization of the stem cell potential of an individual cell is currently not possible, the repeated assessment of the repopulating capacity of different subsets of cells from a certain population will provide a guide to the heterogeneity of cell types, and their relative numbers, within this population. As a result, it is possible to determine the *probability* of a randomly chosen cell from a specified population to exhibit stem cell properties in a particular (assay) system and, therefore, to obtain a measure of the average repopulation potential of this population.

Stem cell potential and behavioral flexibility

Another consensus that was identified during the workshop is that a key feature of HSCs is their ability to *react flexibly* to the current system needs. As a direct consequence, flexibility in cellular action inevitably leads to asymmetries in cellular development. Please note, this does not necessarily imply the existence of asymmetric cell division events (see below for a more detailed discussion), it simply states that cell fate is determined by external as well as internal factors.

Furthermore, if one acknowledges cellular flexibility as critical in defining what constitutes a stem cell, or at least stem cell behavior, it is important to distinguish between the *potential* of the cell to behave in a certain way and the actual *behavior* that is selected. If there are a number of alternative behaviors that a cell may select, it must follow that there is always the possibility that certain behaviors are not actually employed. The question then becomes whether a definition of stem cells should be in terms of *behavior* or *potential*. Let us consider this in more detail and choose between the two options:

A cell is a stem cell if

1. it exhibits stem cell behavior.
2. it has the potential to exhibit stem cell behavior.

Option 1 results in us labeling only those cells as stem cells that exhibit stem cell behaviors in a given, specific assay situation. Option 2, in contrast, implies that testing for stem cells requires us to organize the whole range of potentially very different situations that might induce latent capabilities to be activated. The key point to make here is that the stem cell *potential* of an individual cell can never be determined with certainty, but only in a probabilistic sense.

This distinction between potential and behavior may shed light on some controversial disputes about *stem cell "plasticity"*. The question as to whether a certain tissue specific (stem) cell has the general potential to contribute (by whatever mechanism) to other tissues is considerably different from asking whether it does so in an unperturbed situation. Challenging a specific potential might require artificial and potentially non-physiological manipulations, which are not relevant under normal circumstances, potentially explaining that these phenomena are hardly ever observed in unperturbed systems.

Heterogeneity of stem cell behavior

Both the *potential* of HSCs to behave in certain ways and the actual behaviors that are observed clearly indicate that HSCs form a heterogeneous population. There was general agreement amongst the workshop participants that heterogeneity of HSCs is an important issue when discussing system dynamics both in terms of *potential* and *behavior*. Cellular heterogeneity can be characterized by differences in the (genetically or epigenetically determined) potentials of cells, but also by differences in the actual expression of these potentials. As already mentioned, the flexible challenge of cellular functionalities results from the different systemic needs, which are communicated through the different, local environmental stimuli. Again there was complete agreement that it is the stem cell supporting microenvironments, so called *niches* [18] which play a key role in determining the fate of a stem cell with a given potential. And it would be naive to suppose that all niches are homogenous and elicit the same behavior in all cells with the same potential. Also, there is growing experimental evidence that there is not just one typical HSC niche but rather different types of stem cell supporting contexts [19-23]. There were a number of participants who believed that there is in fact a continuum of these niche milieus just like there is a continuous range of potentials a cell can have. Also, whether a stem cell niche is static or can change its cellular composition and/or functional capacities over time is unclear.

The stem cell hierarchy dogma

HSCs and progenitor cells are classically considered to develop according to a differentiation hierarchy that is characterized by a sequential and irreversible restriction of the fate potential of individual cells [24-26]. This dogma, which is similarly depicted in almost all text-books on HSCs, is still the prevailing view within the scientific community of stem cell biologists. However, if it is a *requirement* that stem cells need to be able to flexibly react to different system needs, it follows that stem cell entities that are restricted to pre-determined developmental pathways would be extremely inefficient. Furthermore, there is an increasing amount of data demonstrating that HSCs can flexibly change properties such as cell surface markers, proliferative activity or repopulation and differentiation efficiency [27-32].

In our view, the stem cell hierarchy is a simplified and discrete view of the role an individual stem cell can take on. It correctly describes the average system behavior on the level of cell populations but it does not adequately represent the regulatory mechanisms at the individual cell level. At this level cells with *stem cell potential* can flexibly and reversibly switch between different roles within the hierarchy depending on system needs.

Reversibility

Although heterogeneity and flexibility of HSCs are widely accepted, it is still an unresolved question whether *reversibility* of functionality and/or phenotype constitutes an essential mechanism of stem cell organization. From the theoretical side there are strong arguments for the necessity of at least some degree of reversibility of stem cell properties. If one assumes that a strict and lossless maintenance of a certain cellular state over time is impossible, reversibility becomes inevitable to guarantee self-maintaining or even self-renewing stem cell systems. Again we must revisit the *potential vs. behavior* view. It is quite possible that a cell adopts a behavior A, then at a later time a new (possible less stem cell-like) behavior B and then reverts back to behavior A. In some sense the stem cell behavior has been reversed. But this may simply be the fact that the cell has potential for both behaviors A and B and the microenvironment challenges the cell in such a way that behavior A, then B, then A ensued. So, even though reversibility is observed at the *behavior*-level, it has not actually taken place at the level of the cells *potential* to act.

However, what if the potential of the cell changed? In other words that it had potential to behave as A or B, then only as B, but then later it regained its potential to behave as either A or B. It would make sense to say that stem cell *potential* has been reversed in a very real sense. The cell has become more ‘stem-like’. Because it is experimentally hard to demonstrate that a cell’s potential became more stem-like over a specified time (see discussion above), it is here where the role of theoretical (quantitative) models is critical. It is models that enable us to systematically investigate (*analytically* or by *simulation*) what happens if potential reversibility is modeled and what happens if it is not. We can’t ever know the potential of living things but we can of simulated ones, and moreover, the way that this impacts on the *behavior* of the entire system.

Asymmetry of stem cell fate

Another point of debate was the occurrence of *asymmetries* in cellular development. Although there was no doubt about the necessity and existence of asymmetries in cellular fate decisions, it is still unresolved as to whether the classical paradigm of an asymmetric stem cell division is a relevant mechanism in the hematopoietic system. This paradigm – which sits alongside the fixed discrete stem cell hierarchy – assumes that the division of a stem cell generates one identical stem cell and one increasingly differentiated cell at some position lower in the hierarchy. Again, it should be noted that the concept of asymmetric stem cell division is still the prevalent explanation for a self-renewing population of HSCs, even though no functionally relevant asymmetries during cell division have been demonstrated in the hematopoietic system to date.

In a strict sense an asymmetric division mechanism as described above would only allow for the self-*maintenance* (in the sense of preserving a population of cells with identical properties), but not for a true self-*renewal* [33]. Self-*renewal*, in the strict sense of the word, implies the re-gaining of something that had previously been lost. If self-renewal refers to stem cell numbers, it requires at least the possibility of an expansion of the stem cell population (such as that which occurs after injury and could arise, for example, by symmetric division with daughter cells both identical to their mother); if it refers to cellular properties then it would even require some sort of reversibility in the differentiation process.

The problem is largely semantic in that the term “self-renewal” is misleading as representing the concept of self-replication.

If stem cell populations are considered (in contrast to the classical view) as continuous, self-organizing systems, any division might be seen as asymmetric. This is because even if the potential of two daughter cells would be identical (which is extremely unlikely in a continuous world) they would still be occupying different spaces and, therefore, necessarily not be identical. Thus, any cell division might be considered inherently asymmetric, due to cumulating small stochastic effects, with the results that both daughter cells will always be different to the mother cell. Such a perspective (proposed e.g. by Mark Kirkland) would then necessarily require some degree of reversibility in potential to afford self-renewal as well as self-maintenance of the population. An alternative concept that has been suggested [34,35] is that any cell division can *a priori* be assumed to be symmetric (neglecting small random differences), with the results of the two daughter cells being identical to the mother cell. Differentiation (in the sense of changing cellular properties) is considered as an *independent* process. This concept of considering cell division and differentiation as (in general) independent processes would allow a consistent explanation of asymmetries in cell fate development that occur during the process of cell division (as e.g. reported for epithelial [36] or neural [37,38] stem cells) as well as of those not linked to cell division events (as e.g. in the hematopoietic system) within the same conceptual context. See Fig. 1 for illustrations of the different concepts.

Niche topology

There was consensus about the existence of different types of niches that interact with HSCs. However, so far there is little information about the dynamics of these interactions. Do HSCs prefer a particular niche under particular circumstances? Are niche characteristics fixed or do they change dynamically? Is there competition of HSCs for particular niches?

Data suggest that the most potent HSCs are deeply dormant in a healthy organisms dividing only very rarely. These dormant cells are distributed as single entities and are often found directly attached to endosteal osteoblasts in the trabecular bone suggesting that niches may only house a single stem cell [32]. There is no doubt that stem cell/niche communications are critical for the control of HSC dormancy and activation on one hand and regulation of the balance between self-renewal and differentiation on the other. Three-dimensional modeling of the physical topology of the stem cell/niche unit will be critical to better understand the complex cell autonomous and non-cell autonomous processes guiding HSC behavior during homeostasis and injury.

Summary

The predominant views of the workshop were as follows:

1. In referring to, or defining “stem cells” it should be specified if the *potential* or the actual *behavior* is considered as the underlying criterion.
2. Stem cell systems should be considered as self-organizing sets of cells in which individual behavior is the result of the potential of the cell and the local environmental conditions. This perspective, however, does not exclude a *simplified* description of stem cell systems as structured populations of (dynamically stabilized) subpopulations of cells at different states if the individual cell level is not relevant.
3. The microenvironment affects the behavior of cells.

4. Stem cell niches are probably heterogeneous (for example, osteoblastic and vascular niche might be different types of niche) – possible continuously so – and the topology of niches affects behavior.
5. At least some degree of reversibility (of cellular properties/functionalities) is necessary to achieve true self-*renewal* within a continuous view of the world.
6. Asymmetries in individual cell fate development should be described by considering cell division and differentiation as independent processes. This view is more consistent with the entire range of experimental observations than the prevailing view of asymmetric division events. However, again the latter perspective is a possible simplification if considering the cell population level alone.
7. The range of stem cell potentials and their microenvironments are continuous and any 'discrete' description is necessarily a simplification.

Modeling CML

Mechanisms causing leukemic abnormalities

CML is caused by a t(9;22) chromosomal translocation leading to the formation of the BCR-ABL oncoprotein. There are a number of molecular processes and signaling pathways, related to proliferation control, apoptosis regulation, as well as cell adhesion and mobility that are known to be affected by BCR-ABL (for a review see [39]). It is generally accepted that as a result of the oncogenic activity, BCR-ABL-positive hematopoietic stem/progenitor cells can be characterized by one or more of the following properties:

i. an *elevated proliferative activity*;
ii. a *decreased apoptotic activity*; and/or
iii. an *altered stem cell – microenvironment interaction (e.g. stroma adhesion behavior*).

Key to understanding CML is to know how these properties at the individual cell level affect the systemic level. It is unresolved as to how the altered individual cell behavior influences the cell–cell and cell–microenvironment interaction in such a way as to produce the tissue organization as a multi-cellular phenomenon that we can observe. Furthermore, it is not known which of the altered cellular properties is the driving force that leads to clonal expansion and to the malignant overgrowth of the system by leukemic cells. If we knew this, we would have a better understanding of why treatments worked in the way they did, and propose improve treatment methods as a result.

During the workshop a number of different possible mechanisms leading to a leukemic system have been presented and discussed. First, it was suggested that a difference in cell kinetic properties, such as increased proliferative activity or decreased apoptosis, is not necessary to explain leukemic expansion. In other words simply item (iii) in the list above would suffice. Simulation results presented by Janis Abkowitz [40] showed that the use of additional stem cell supporting niches by BCR-ABL-positive cells would be sufficient to explain dominance of the leukemic clone in the system. An alternative model, presented by Ingo Roeder [41], demonstrates that a relative advantage of BCR-ABL-positive HSCs to utilize available stem cell supporting niches is sufficient to induce an ultimate leukemic conversion of the system. This model does not require additional niche space. However, Roeder et al. demonstrated that without the additional assumption of some difference in cell kinetic properties (such as an enhanced proliferative activity), the clinically observed increase in total cell production could *not* be explained. Even though the model results of Abkowitz et al. and of Roeder et al. both suggest an altered cell–niche interaction as a likely

mechanism to explain the competitive advantage of the leukemic clone, they do not (formally) exclude other possible reasons. Though these two speakers propose slightly different mechanisms, they agree that BCR-ABL-positive HSCs need to exhibit a competitive advantage relative to the normal cell population to explain the clinical observations. This result was also substantiated by the mathematical model of CML proposed by Franzika Michor et al. [43,44]. This model uses differential equations and is therefore at a higher level of abstraction than the others discussed and so remains neutral about the mechanisms taking place to induce the growth advantage. However, their results also demonstrate the necessity for normal HSC homeostasis to be altered for leukemic cells in such a way that they exhibit a clear competitive advantage over normal cells.

The origin of CML

Another frequently discussed issue at our workshop concerned the origin of leukemic activity. There was agreement that the development of manifest leukemia requires leukemic cells to have stem cell potential. In other words, there must be one or a small number of affected (i.e., mutated) cells that are able to generate a population with sufficient numbers to maintain leukemic hematopoiesis in the long-term. However, the origin of these initial LSCs is still under debate.

The most obvious candidate explanation is that the leukemic transformation (i.e. the induction of a competitive advantage) is induced in the population of HSCs. Alternatively, another explanation is that cells in more differentiated stages might be mutated and then (either immediately or later) transformed into a cell type with increased stem cell potential. Whereas the result – an LSC – is the same in both perspectives, the originating cells might differ. In fact in a continuous view of the world there is no real qualitative difference between these views — it would come down to which cells in which environmental conditions are more or less likely to be transformed. However, in keeping with a more discrete model perspective it was proposed that we need to distinguish between *LSCs* and *leukemia-initiating cells*. In this view, leukemic stem cells are the result of the initiating process. The first option is that they are derived from non-stem cells by inducing stem cell potential together with the leukemic transformation. The second option is that they result from HSCs by the induction of leukemic properties that do not change their stem cell potential.

Most theoretical models of CML discussed during the workshop (presented by Janis Abkowitz [40], Franziska Michor [43-45], Dominik Wordarz [9], Ingo Roeder [41,46] and Peter Kim [47]) assumed the existence and/or induction of leukemic activity in the HSC population without considering leukemia induction at other cell stages. The second option was proposed by Mark Kirkland (unpublished data) who argued that through the disturbance of the (erythroid) differentiation process, the population of early (erythroid) precursor cells might experience a dramatic expansion. Kirkland's model requires a very small probability of these cells to de-differentiate and become stem cells. He proposes that leukemic transformation might occur at the level of precursor cells (that is cells that have lost stem cell potential but are not yet fully differentiated), generating a huge expansion of such cells. Because there are now so many transformed precursor cells, even though the probability for any individual cell is very low, there is now a significant *system* probability for de-differentiation to take place, which in turn affects the stem cell population.

The question as to whether or not a particular BCR-ABL-generating mutation leads to a manifest leukemia is impossible to answer in general. It may be the case that there are such cells in every human that are kept under check, or that it takes only one cell, and some other specific environmental circumstance, to produce manifest leukemia. Screening data on healthy volunteers shows that BCR-ABL positive cells can be detected in healthy people,

even though at a very low frequency [48-50]. Such observations would be consistent with a stochastic description of CML development as suggested e.g. by Abkowitz et al. or Roeder et al. Both models predict that the growth advantage induced by the leukemic transformation would result in an overt leukemia only in a minority of patients because there is a significant probability of those cells to differentiate or die instead of generating a stable leukemic clone.

Modeling treatment dynamics, residual disease (disease persistence) and treatment resistant clones

As imatinib treatment currently represents the gold standard for treating CML, this therapy was also in the focus of the presented modeling approaches. A typical characteristic of the disease dynamics under imatinib is a bi-phasic decline of BCR-ABL transcript levels (i.e. a steep decline within the first 6 months of treatment followed by a more attenuated decline), which is observed in the majority of patients. This dynamic behavior had first been addressed in a mathematical model proposed by Michor et al. [43] who explained the imatinib response in CML patients up to one year after treatment start. Later the model description of the imatinib response had been extended to the description of 5 year follow-up data [41,51].

Although imatinib (and in general TKI) treatment is able to efficiently reduce the numbers of leukemic cells in the majority of patients, clinical data show that a considerable amount of *residual* leukemic cells can be expected to survive for prolonged times. There are several candidate mechanisms that can be proposed to be responsible for this persistence:

- **i.** treatment-resistant LSC, either existing before or newly generated after treatment starts
- **ii.** some LSCs are in a particular state (or microenvironment) that hides them from treatment
- **iii.** some leukemic cells are not sufficiently in contact with the drug (achieve an intracellular concentration) for their behavior to be altered
- **iv.** there is a sufficiently high number of leukemic cells each with sufficiently high BCR-ABL protein levels to neutralize the affects of TKI treatment.

This list suggests we distinguish between *residual disease* (collection of diseased cells left after treatment), and selective *treatment resistance* (specific cells that are not able respond to treatment). Note that residual disease may or may not include resistant clones.

As shown by long-term follow-up data of patients under imatinib treatment the number of residual leukemic cells declines slowly for the majority of patients, at least as long as no treatment resistance occurs. This data provides strong evidence that imatinib treatment affects the LSC, a conclusion that is supported by different modeling approaches (see [11] for a review). Although the mechanisms and the degree of this effect are still under discussion, there was consensus that a certain reduction or at least a decreased expansion of the LSC pool has to be assumed as a result of the imatinib treatment to explain clinical observations.

It is also widely accepted that the imatinib effect on LSC spares quiescent (i.e. non-proliferative) cells [52,53]. This specificity of the treatment effect is most likely one of the major reasons for the long-term persistence of residual LSC, even without the existence of definite treatment resistance that would arise through mutations of the ABL kinase domain of the BCR-ABL protein, for example.

As a result of the limited sensitivity of current PCR methods, the exact number of residual leukemic cells is difficult to predict once it has fallen below a certain level (Fig. 2). Therefore, current estimates of the long-term BCR-ABL dynamics and also of the time to a potential eradication of all leukemic cells are purely based on model predictions (see e.g. Roeder et al. [41]) and are still lacking validation by clinical data.

Patient heterogeneity

Although a continuing decline of the BCR-ABL levels over 5 to 7 years could be demonstrated as an average effect across CML patients, there is a considerable heterogeneity of this effect among patients. Particularly with respect to treatment decisions after achieving undetectable BCR-ABL levels, a better understanding of patient specific kinetics is necessary. Even though in some cases the level of BCR-ABL is undetectable for some years after stopping treatment [54], we cannot be sure whether or not stopping TKI treatment in general is a safe option.

As suggested by a simulation study (presentation by Matthias Horn and Ingo Roeder) the best predictor for the probability of a molecular relapse and the reoccurrence of the disease after treatment cessation, is the absolute number of residual BCR-ABL positive stem cells. Because this number is not available from clinical data, it will be important to obtain reliable estimates for it from other, clinically observable parameters, such as the BCR-ABL decline kinetics in the blood within individual patients. In this respect, particularly the long-term BCR-ABL decline kinetics (i.e. the second part of the bi-phasic decline kinetics), is predictive, because it represents the treatment response of the residual (most likely quiescent) LSCs. However, there is the hypothesis that also the initial treatment response (i.e. the BCR-ABL kinetics during the first 6 months after imatinib treatment initiation) might be predictive for the residual disease kinetics. To test whether or not this hypothesis is true, a meta-analysis of individual imatinib-response kinetics across different clinical trials was proposed at the workshop.

Treatment resistance and clonal heterogeneity

Patient heterogeneity can have a number of different determinants. A major factor that has been extensively discussed is clonal composition, i.e. the existence of subpopulations of leukemic cells with different growth kinetics and/or treatment sensitivities, generated by the alteration/mutation of the properties of a single (leukemic) cell that is preserved in its progeny. Different mechanisms leading to *treatment resistance* and/or *disease persistence* (see above) have been described: e.g. intracellular TKI concentrations due an increased drug efflux or due to TKI degradation, different levels of the oncoprotein e.g. due to over-expression and amplification of the BCR-ABL locus, the activation of alternative regulatory pathways, general pharmacokinetic resistance, and mutations in the ABL kinase domain that inhibit the binding of TKI molecules (see e.g. [55] and Fig. 3). All these mechanisms might lead to a heterogeneous composition of the population of leukemic cells with different degrees of treatment sensitivity.

Some of the mutations in the ABL kinase domain can have severe manifestations with the result of a complete TKI resistance of these cells. Why and when such resistance mutations occur is still under debate. Also, the mathematical modeling approaches discussed during the workshop did not lead to a unique conclusion. Whereas the model presented by Dominik Wodarz [9,56] suggests that most (if not all) resistance mutations already exist at therapy initiation, the models proposed by Franziska Michor [43,44] and Ingo Roeder [41,46] lead to consistent results also for the assumption of resistance mutations that arise after therapy initiation.

Another open question in the context of clonal heterogeneity is whether or not the expansion of a leukemic clone can be affected by other *competing clones*. For example, there are reports that the BCR-ABL translocation can be found in a considerable number of healthy people and there is a sensible hypothesis that a manifest leukemia will only occur in a small minority of these cases [48,49]. It is currently unclear whether these leukemic clones disappear because they arise in short-term contributing (e.g. precursor) cells or whether they are LSCs whose clones might go extinct due to the competition with normal stem cells or whether they are eliminated by an immune response that fails in the few patients who develop CML.

Assuming a competition of normal and LSCs, the probability for a manifest leukemia or similar myeloproliferative disorders arising from a single cell mutation has been estimated on the basis of model simulation to be in the range of 15% [40] to 20% [41]. If there is a competition of different clones for common limited resources (such as stem cell supporting “niches”), this might also explain the dynamic changes in the size of different leukemic clones under changing treatment. For example, there are observations suggesting that reducing the size of treatment-sensitive leukemic clones might cause or enhance the expansion of particular treatment-resistant clones [57]. In the context of clonal competition such an observation can be explained if one assumes that treatment-sensitive clones are limiting the expansion of the treatment resistant clones as long as they have a certain critical size. This limiting competition effect, however, might be lost if the number of treatment-sensitive cells is reduced sufficiently. To resolve the question as to whether clonal competition and selection mechanisms can consistently explain the clinical observation and under which circumstances they might become relevant, will be an important target for further model analysis.

Treatment options for CML

TKI treatment optimization

Although TKI treatment can be regarded as a highly efficient therapeutic option, treatment resistance and persistent residual disease still represent major clinical challenges. Beyond the development and the clinical testing of next generation TKIs, which more efficiently target the whole spectrum of leukemic clones, the optimization of current TKI treatments is an important scientific target. It will be necessary to optimize treatment schedules that *combine different TKIs* by balancing potential side effects and treatment efficiency.

A second possibility is the *combination of TKI treatment with cytokines*. One rationale of such a strategy is based on the observation that imatinib selectively spares quiescent cells. As suggested by *in vitro* data [58] and by simulation results [41], the activation of quiescent LSCs into cell cycle using e.g. the cytokine G-CSF might have the potential to increase the efficiency of imatinib treatment. However, clinical testing of a sequential application of imatinib and G-CSF in 28 day cycles (21 days imatinib followed by a 7-day imatinib gap with 3 pulse doses of G-CSF at days 24, 26 and 28) did not show any advantage of this combination compared to imatinib alone [42]. Although this result seems at first glance to be discouraging, simulation studies based on the model proposed by Roeder et al. (unpublished data, presented at the workshop by Matthias Horn) show that the predicted beneficial effect depends on the application schedule: the model predicts that it is strongest for a simultaneous application of the proliferation activating cytokine and imatinib but it is almost lost for a sequential application of imatinib and G-CSF. Critically, the pilot trial contained exactly such a sequential application. Therefore, it still needs to be resolved as to why a simultaneous application of G-CSF and imatinib did not show any effect in the *in vitro* setting, which motivated the clinical trial and whether the predicted benefit of a simultaneous application can be achieved in vivo.

Another promising option is the combination of TKI and interferon (IFN)-α treatment. Chronic treatment of IFN-α has a negative effect on the self-renewal capacity of mouse HSCs *in vivo*, acute IFN-α treatment results in the very effective activation of dormant HSCs. Most interestingly, IFN-α activated HSCs show a dramatically increased sensitivity to anti-proliferative chemotherapy mediated killing [59]. Although it remains to be shown whether human HSCs or LSCs behave similarly, these results open the possibility that in a scheme similar to the one mentioned above for G-CSF, IFN-α may be used to activate, dormant CML stem cells and thus sensitize ("prime") them to imatinib mediated elimination. In agreement with this hypothesis, some patients initially treated with IFN-α but subsequently switched to imatinib (due to the change in the treatment protocol) showed a surprisingly high rate of long-term complete remission (>2 years) [59]. These data suggest that the cooperative effect of IFN-α "priming" with imatinib may create a possibility to target the CML-stem cell.

Alternative drug treatment

As quiescent LSCs are able to "hide themselves" or be insensitive in some other way to TKI treatment, the use of compounds that are able to target specifically this population of cells obviously suggests an alternative approach. BMS-214662, a cytotoxic farnesyltransferase inhibitor that was reported to kill non-proliferating tumor cells, has been suggested as a potential compound for an effective treatment of CML [60]. BMS-214662, alone or in combination with imatinib or dasatinib, was shown to induce apoptosis of both proliferating and quiescent leukemic stem-progenitor cells *in vitro* whereas normal stem-progenitor cells were relatively unaffected. Although these results still need to be consolidated, a selective apoptosis induction in quiescent LSCs can be expected to enhance the reduction of residual LSCs.

Immunotherapy

The potential cure of CML patients by allogeneic stem cell transplantation relies to a considerable degree on the graft-versus-leukemia effect, which consists of the immunological recognition of antigens on residual leukemia cells by donor T lymphocytes. The idea of immunotherapeutic approaches is to take advantage of a similar effect for patients who do not receive a stem cell transplantation, applying vaccines which target leukemia-specific or leukemia-associated antigens to eradicate residual leukemic cells. Although, targeting leukemia-specific BCR-ABL peptide vaccines did not show the expected efficiency (Michael Deininger, unpublished data), peptide vaccines targeting leukemia-associated antigens which are overexpressed in CML cells (e.g. WT1 and PR1, derived from Wilm's tumor-1, and proteinase 3 and elastase proteins, respectively) have been shown to be efficacious and safe [61-63]. In order to more efficiently target residual disease in the majority of chronic phase CML patients currently treated with TKIs, a combination vaccine of WT1 and PR1 peptides is suggested as this has the ability to target both primitive and mature CML progenitors [64]. Despite these experimental results that clearly point to the general potential of immunotherapeutic approaches in CML treatment, it is also the case that current mathematical models predict the potential of vaccination to ameliorate the therapeutic outcome of TKI treatments. Based on the clinical data, showing that there is an inherent immune response in a considerable part of imatinib-treated CML patients [65], Kim et al. [66] investigated the dynamic system response of a vaccination in these patients using a revised version of the mathematical CML model proposed by Michor et al. [43]. Their simulation results demonstrate that vaccination (using inactivated CML cells) might be able to amplify the inherent immune response and to drive the residual LSCs into extinction.

On quantitative modeling

Quantitative (mathematical) models help us – sometimes in an abstract way – to understand the nature of things that we observe. But there are some key properties of models that often go overlooked and it is worth enumerating them here. Our main purpose within this section is to increase the role and significance of modeling in the view of those who traditionally have not used models. We therefore provide a list of issues that should be considered when developing models with a view to encouraging interdisciplinary collaboration and gaining the "buy in" of clinicians.

1. Models are always a simplification of the (physical) world they represent. However, simplification and abstraction is often the key to understanding generic principles and thus providing biological insights.
2. Models are artificial, and whilst we have ways of tying them with reality (such as falsification) they can never have the same reality as experiments. Nevertheless, they can identify inconsistencies in experimental data and/or their interpretations.
3. Models always encode assumptions about the world, and any implicit assumptions should always be made sufficiently clear. Also, it should be made clear how the model "copes" when any of these assumptions are relaxed.
4. There is no unique ("best") model for a particular problem. Therefore, for any model its assumptions, its scope, its limitations, and its significance to experimentalists should clearly be outlined.
5. A model is always caged in particular mathematical method, and any method has advantages and disadvantages over other mathematical methods.
6. Models should demonstrate consistency with real sets of data and, furthermore, point to new experiments to determine possibly unexplored or undetermined potential behaviors. In other words, models should be testable and predictive.
7. Models should be flexible and extensible allowing new ideas, concepts and theories to be incorporated.

Beyond fitting data and proposing testable predictions, modeling has a role in proposing new hypotheses and, therefore, in changing commonly held out-dated views. This is particularly true for the demonstration of inconsistencies of "old" paradigms as well as for providing alternative explanations. With respect to stem cell biology, we think that mathematical models (including those presented at the workshop) will help to resolve general inconsistencies in explaining e.g. the hierarchical appearance of stem and progenitor cell populations on the one hand and the observed flexibility and reversibility of stem cell functionality on the other hand. One way to achieve this is to describe stem cell populations as dynamical (self-organizing) systems, rather than as fixed, pre-determined structures.

As outlined above, there is no unique model representation of a biological process. In this respect it is interesting to note that several of the models (discussed at the workshop) use different ways to describe cellular development in the hematopoietic system. Some approaches comprise four distinguishable cell types (stem, progenitor, precursors and mature; e.g. Michor, Kim), whereas others consider only stem and non-stem cells with a structured stem cell population (e.g. Roeder) or even a complete continuum of cells with varying stemness potential (e.g. Kirkland). To compare these approaches, it is necessary to, firstly, be very clear about language (e.g., what defines stem, progenitor, precursor or mature cells) and to, secondly, outline the scope of the particular model. We have discussed the definition of stem cells in previous sections. Progenitor cells are cells that can be isolated from hematopoietic tissue (marrow) and grown in various culture systems as clonal colonies

but that lack long-term *in vivo* repopulating ability. Whereas multipotential progenitors can undergo differentiation into several lineages (as judged by the presence for example of megakaryocytes, neutrophils, and erythrocytes in the colony), unilineage progenitors can generate only a single lineage (e.g. erythrocytes or neutrophils or monocytes, etc). In contrast to the more primitive progenitors, precursor cells can be identified by light microscopy of marrow and their kinetic behavior has been described in extenso. For example, they range from proerythroblasts to orthochromatic erythroblasts, myeloblasts to band neutrophils, etc. Mature blood cells are self-evident.

Whereas heterogeneity of hematopoietic cells is represented in terms of subpopulations by some of the models, it is described by continuously varying cellular properties by others. None of these descriptions claims to be exclusive, but they focus on different aspects and one way to understand general biological principles is to identify those bits and pieces of the different model descriptions which represent conceptual differences and are not simply alternative ways of a mathematical representation. One candidate of such a “generic” principle is *reversibility* of stem cell properties.

Also with respect to the applied mathematical methodology the presented models differ; two dominant modeling strategies arose: The first of these uses deterministic *differential equations* and gives a high level analysis of the global properties of a stem cell system [43,44,47,66]. Such models are attractive as they can be tuned to fit data at the cell population level and because they are reasonably straightforward to specify. Such a representation of stem cell populations approximates the system behavior by describing the “average” behavior of cells. That is to say, these models are correct at the cell population level, but cannot describe individual cell fates. The second general class of methods includes *stochastic processes* into the description. Although there are different roles for stochastic components in mathematical models, they are essentially placeholders for where we do not have enough information to model the specific physical (and specifically causal) processes that are taking place. In contrast to the above mentioned deterministic differential equation models, they allow for a description of the heterogeneity within cell populations. Whereas some of the stochastic approaches are acting at the cell population level [40,56,67], others are explicitly modeling the fate of individual cells and allowing for a representation of the clonal relationship of all cells within the system [41,68].

Stochastic representations, as they have been applied here, stand for an approximation of complex cellular and molecular interactions. However, as we gain further information about the subject we are modeling, we may begin to model our cells and their environments in more detail. As we do, we can start to remove stochastic approximations and to model the internal state of the cells and the external environment by (simple) physical or chemical mechanisms. This relatively new method of modeling complex systems is known as an *agent-based modeling* [69]. The advantages of this approach are that we can begin to investigate how specific chemical and spatial mechanisms at the level of individual cell behavior can affect the system behavior and that they are not biased by any mathematical technique [70]. Agent-based modeling is a specific approach to thinking about dynamic systems, one that combines simple mechanisms with computational processing to generate complex outcomes or phenomena. Moreover, in situations where individual components within a living system cannot be observed without damage or change to those components (such as changing the environment), the agent method allows one to work in simulation, with precise observation of experiments at multiple levels, something currently unachievable in wet-lab experiments. In years to come it seems clear that this approach will increase in popularity.

Thus, it is difficult to say what makes a good model in any general sense, but important issues definitely include *simplicity* so it can be analyzed and understood, *clarity* so that the assumptions are made clear, *accurateness* so that it matches data, *computability* so that we can simulate complexity, *flexibility* so that it can be applied to more than one set of experiments, *robustness* in that small perturbations do not impact catastrophically on self-organization, and *testability* such that the model can be validated/falsified by comparison with real data. Also, it should be emphasized again that models are always *hypotheses* (even if they consistently describe real data) and that different modeling approaches are often promoting conflicting perspectives. However, these facts do not disqualify models as serious scientific tools. In contrast, it is one of the strengths of mathematical models that they allow us to investigate, test, and compare different alternative hypotheses. In particular, the investigation of alternative perspectives is vital for the scientific process to arrive at a more comprehensive understanding of complex biological processes.

A summary and the way forward for CML research

From the workshop discussions two leading, clinically relevant, questions emerged. First, *is there a predictable time of cure under current TKI treatment scenarios?* Second, *what is the optimal strategy to target LSCs?* Further open questions that were discussed on the basis of the presented concepts were the origin of CML, the resistance dynamics and mutation evolution of cells, models of treatment dynamics, as well as making predictions of new treatment options for CML. Conceptual assumptions that were under debate could be listed as follows:

1. Is the concept of reversibility of stem cell properties/functionality necessary?
2. Do we have to distinguish between progenitor and stem cells, as the target cell population (leukemia-initiating cells) for leukemic mutation events?
3. What differences of leukemic and normal stem cells have to be assumed? Is there a cell specific growth advantage or disadvantage and/or is there a competition for resources, such as niche space?
4. Should the cell kinetic status of leukemic (stem) cells be considered as a relevant factor effecting treatment efficiency?

There was agreement that besides their application in identifying general mechanisms of disease development and therapeutic effects, a major potential of modeling approaches in the field of leukemia research is their application with respect to the design of optimized treatment schedules, including the *model-based design of clinical trials*. As demonstrated during the workshop, this is not only a potential option for the future, but it is a tool which is available *right now* as we have mathematical models available that already provide qualitative ideas as well as tangible quantitative predictions for particular treatment scenarios (e.g. [41,43-44,47,68]).

There are a number of issues, identified during the workshop that should be investigated (experimentally and theoretically) in the future. One important field is a better understanding of clonal competition effects between different (leukemic) stem cell clones. Furthermore, it became clear that immunological effects should not be neglected in the modeling approaches because they might sensitively affect the clonal dynamics of leukemic cells. We also identified the need for a better characterization and understanding of the property differences between normal and LSCs. As the knowledge of cellular parameters (e.g. proliferative activity or chemosensitivity) has considerable effects on the choice of model assumptions and, therefore, on the predicted system dynamics, it would be preferable if experimental studies were planned in close collaboration of experimentalist and modelers to ensure the correct determination of most relevant and informative parameters. This similarly

applies to clinical investigations. The workshop demonstrated the potential that theoretical methods can have in predicting possible quantitative effects that might be expected under certain conditions, and it is increasingly the case that model analyses and simulation studies should be taken into account as an additional/complementary means when designing clinical trials.

Arguably the greatest success of this workshop was to bring clinicians, experimentalists and modelers together. Clinicians and experimentalist are beginning to realize the value that theoretical models can contribute to the understanding of biological processes and to the optimization of therapeutic strategies. On the other hand, modelers gained a better insight into practical problems and constraints of experimental and clinical investigations. We believe that this workshop has contributed to paving the way to more fruitful synergy in the future modeling of CML.

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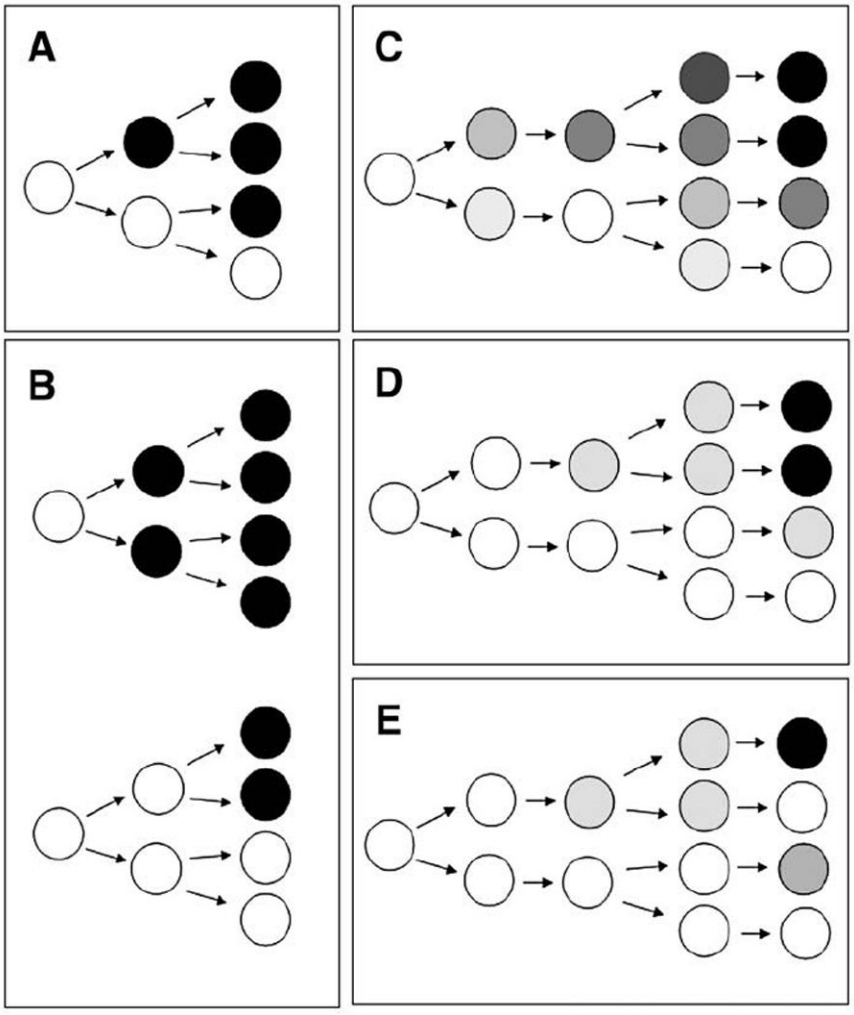

Fig. 1.

Concepts of stem cell fate asymmetry. Unfilled circles: stem cells; Filled circles: Differentiated cells (different grey levels illustrate different degrees of differentiation) (A) Strictly asymmetric stem cell division at the individual cell level. (B) Two options of (strictly) symmetric types of cell division at the individual cell level, which allows an explanation for asymmetric stem cell fate at the population level. (C – E) Assumption of a cell cycle independent differentiation process. (C) Assumption of an inevitable (small, stochastic) asymmetry in every cell division. Differentiation process assumed to be reversible, which allows for self-maintenance (or self-renewal) of stem cell state and of continued differentiation. (D, E) Assumption of strictly symmetric cell divisions (potentially as approximating small, stochastic differences) (D) Differentiation independent of cell division but irreversible (only self-maintenance of a cellular state but no self-renewal possible). (E) Differentiation independent of cell division but potentially reversible (both self-maintenance and self-renewal of a cellular state possible).

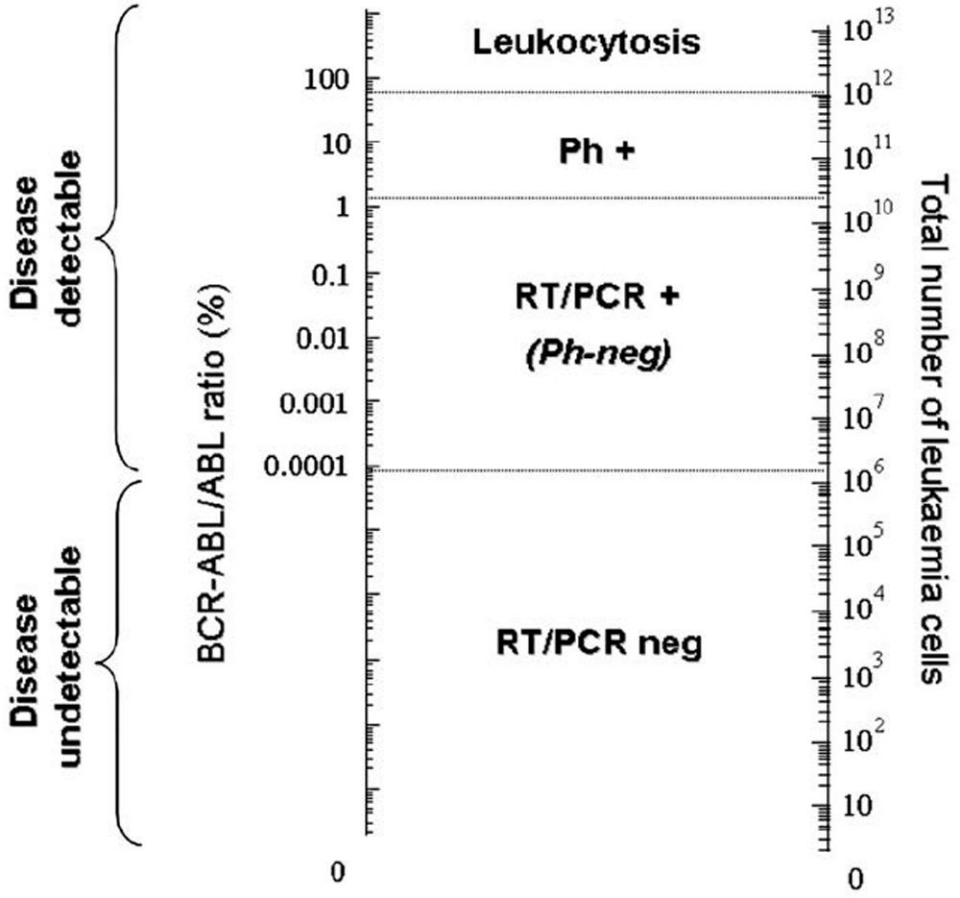

Fig. 2.
Relationship of absolute number of leukemic cells and BCR-ABL transcript levels (adopted from a scheme provided by Junia Melo.)

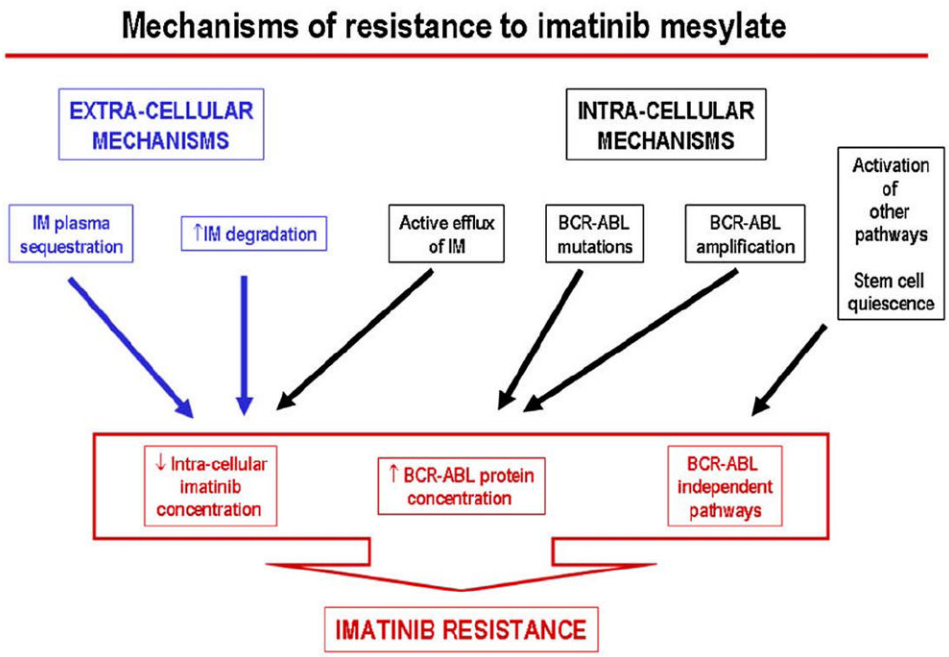

Fig. 3.

Mechanisms of imatinib mesylate resistance (adopted from a scheme provided by Tessa Holyoake). The scheme shown a collection of mechanisms that have been suggested as to potentially inducing resistance of leukemic cells to imatinib mesylate treatment.