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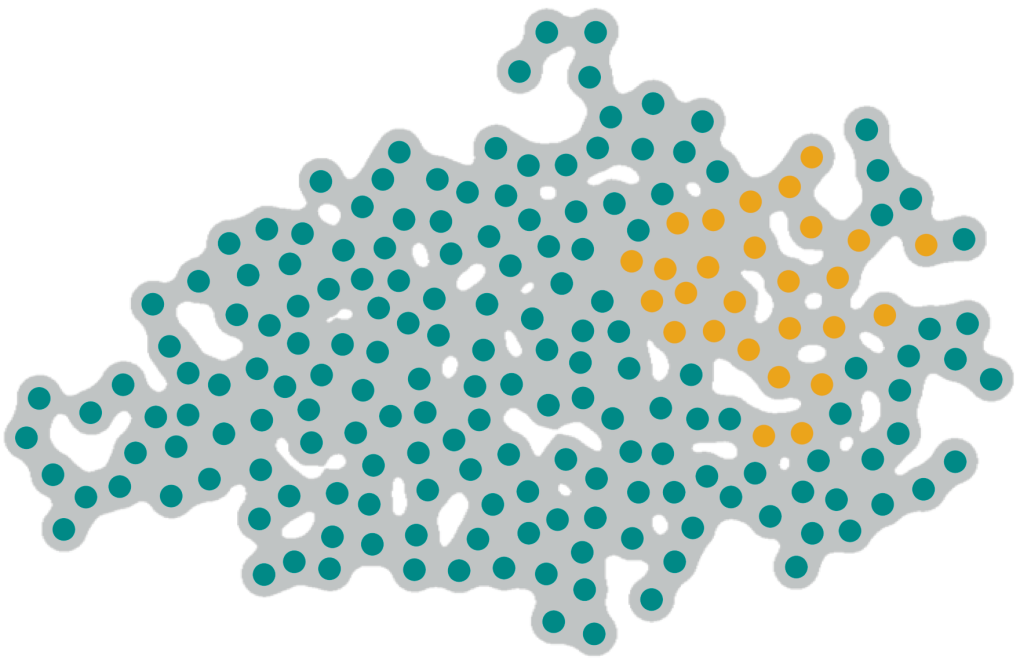
Jonathan Lindström

Modelling the evolution of treatment-induced resistance in Ph+ leukaemias



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Abstract

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Targeted therapies are a mainstay of modern cancer treatments. Rather than harming rapidly dividing cells in general, targeted therapies work by directly interfering with oncogenic molecular pathways present in a tumour. Consequently, a targeted therapy typically has less severe side effects. However, specificity comes at a price as comparatively small changes to the target can render the treatment ineffective. Much like the natural selection among plants and animals, individual cancer cells compete with one another for space and resources. Hence, if a single cancer cell acquires a resistance adaptation, the forces of evolution can turn that advantage in a single cell into an untreatable resistant cancer.

This thesis is principally concerned with chronic myeloid leukaemia (CML), characterized by a chromosomal translocation called the Philadelphia chromosome which creates the constitutively active tyrosine kinase Bcr-Abl1. The discovery of tyrosine kinase inhibitors (TKIs) targeting Bcr-Abl1 greatly improved treatment outcomes. Eventually however, resistance emerges. An important mechanism in CML is mutations in the kinase domain of Bcr-Abl1 that affect how well the drugs bind. A number of drugs have been developed that target the mutated kinase to varying degrees, but it is still desirable to prevent drug resistance from occurring in the first place, as the accumulation of multiple mutations is almost certain to create untreatable resistance.

The fitness effects of a drug resistance adaptation depend on the drug treatment, so it may be possible to alter the fitness landscape by modifying the treatment. This work examines different approaches, mainly in CML, to delay or prevent the onset of resistance through modifying the treatment protocol.

Periodically switching between different TKIs, i.e. drug rotations, was shown through modelling to increase the expected time to resistance and seems to have some protective benefits in vitro. Also apparently promising were drug combinations involving a novel inhibitor asciminib, currently in phase III trials, which can reduce overall drug burden while also being seemingly effective against known resistance mutations. Finally, a model of the interaction between resistance mutations and less potent alternate resistance mechanisms revealed how a drug holiday may have resensitizing, or even beneficial effects.

Keywords: Chronic myeloid leukaemia, Stochastic modelling, Tyrosine kinase inhibitor, Drug resistance, Clonal evolution

"Some of you may have had occasion to run into mathematicians,
and to wonder therefore how they got that way."

–Tom Lehrer

Populärvetenskaplig sammanfattning

Kronisk myelotisk leukemi (KML) och ett par relaterade åkommor orsakas av Bcr-Abl1, ett abnormalt enzym. Det bildas genom en oönskad mutation och finns praktisk taget enbart i cancerceller. Därifrån aktiverar det en myriad tillväxtprocesser och ger upphov till snabbt växande celler som inte följer kroppens regler: Cancer. Att det drivande enzymet endast finns i cancerceller, och att det är ensamt ansvarigt för att orsaka KML gör att mediciner som hindrar just Bcr-Abl1 är väldigt effektiva behandlingar. Dessutom fungerar de utan att påverka kroppens egna celler.

Sådana mediciner har revolutionerat behandlingen för KML, men framgången störs av återkommande problem med läkemedelsresistens. Cancerceller som tolererar behandlingen bättre än snittet förökar sig mer, och efter en tid återstår bara resistenta celler. Då fungerar behandlingen inte längre. Det kan liknas vid hur naturligt urval ger upphov till nya arter. Men i det här fallet kan vi kontrollera behandlingen. Därigenom borde det gå kunna styra evolutionen i cancercellerna, och således styra hurvida resistens utvecklas.

Med hjälp av simulationer undersökte vi hur olika typer av behandling påverkar risken för resistens. Till exempel så visar det sig att det kan vara positivt att växla fram och tillbaka mellan två olika mediciner. Det sker på grund av de olika behandlingar som finns i dagsläget inte alltid påverkas likadant av anpassningar i cellerna. Med andra ord så kan cancerceller vara resistenta mot en medicin samtidigt som de inte tål en annan. Genom att växla behandling stör man då utvecklingen av resistens; en anpassning kan fås att växla mellan att vara fördelaktig eller värdelös för cancercellen och det saktar ner evolutionen. En annan behandlingsmetod som också verkar kunna ha en skyddande effekt var kombinationer av vissa läkemedel, särskilt en som ännu inte finns på marknaden. Vi visade även att ett kort behandlingsuppehåll kan bidra till att göra cancercellerna mer känsliga för behandling under vissa omständigheter.

Sammanlagt talar denna avhandling för att det går att använda de mediciner vi har för behandling av KML på ett smartare sätt än vad som görs i dagsläget. Genom att utnyttja strategier som att växla mellan, eller kombinera, behandlingar går det både att minska risken för resistens och begränsa besvär med bieffekter.

List of Publications

This dissertation is based on the following peer-reviewed publications:

I Lindström H.J.G., de Wijn, A.S. and Friedman R. Stochastic modelling of tyrosine kinase inhibitor rotation therapy in chronic myeloid leukaemia. *BMC Cancer*, 19:508, May 2019. doi:10.1186/s12885-019-5690-5.

III Lindström H.J.G. and Friedman R. The effects of combination treatments on drug resistance in chronic myeloid leukaemia: an evaluation of the tyrosine kinase inhibitors axitinib and asciminib. *BMC Cancer* 20:397, May 2020. doi:10.1186/s12885-020-06782-9.

Additionally, it includes the work in the following manuscripts:

II Lindström H.J.G. and Friedman R. Rotating between ponatinib and imatinib temporarily increases the efficacy of imatinib in a cell line model. 2020.

IV Lindström H.J.G., de Wijn, A.S. and Friedman R. Modelling resistance in leukaemia mediated by mutationsand alternate mechanisms – their interactions and treatment-free periods (drug holidays). 2020.

V Lindström H.J.G. and Friedman R. Inferring time-dependent growth rates in cell cultures undergoing adaptation. 2020.

For the sake of narrative flow, the enumeration of publications and manuscripts is non-chronological.

List of Abbreviations

ABC	approximate Bayesian computation
ABCB1	P-glycoprotein 1
ABCG2	ATP-binding cassette super-family G member 2
ABL1	Abelson tyrosine kinase
AML	acute myeloid leukaemia
ALL	acute lymphoblastic leukaemia
AP	accelerated phase
allo-HSCT	allogenic haematopoietic stem cell transplant
ATP	adenosine triphosphate, an energy carrier molecule
Ba/F3	an interleukin-3 dependent cell line
BC	blast crisis
BCR	breakpoint cluster region
BP	blastic phase
BCR-ABL1	Philadelpha chromosome associated oncoprotein BCR-ABL1
CI	combination index
CML	chronic myeloid leukaemia
CD34	surface glycoprotein found on haematopoietic stem cells
CP	chronic phase
CSC	cancer stem cell
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum

GIST gastrointestinal stromal tumour

HSC haematopoietic stem cell

IC_{50} 50% inhibitory concentration

IFN–α interferon alfa

IL-3 interleukin 3

KCL-22 a BCR-ABL1 positive CML cell line

KD kinase domain

c-KIT tyrosine-protein kinase KIT

K562 a BCR-ABL1 positive CML cell line

K812 a BCR-ABL1 positive CML cell line

LYN tyrosine-protein kinase LYN

MAPK mitogen-activated protein kinase

hMLH1 human mutL homologue 1

MMR major molecular response

MPAL mixed-phenotype acute leukaemia

MTS tetrazole dye

NADH nicotinamide adenine dinucleotide, an energy carrier molecule

NGS next generation sequencing

OCT-1 organic cation transporter 1

PB peripheral blood

PCR polymerase chain reaction

PDE partial differential equation

PDGFR platelet derived growth factor receptor

Ph Philadelphia chromosome

Ph+ Philadelphia chromosome positive

PSSM position specific scoring matrix

P210$^{\text{BCR-ABL1}}$	a certain BCR-ABL1 isoform associated with CML
RCC	renal cell carcinoma
ROS	reactive oxygen species
SRC	proto-oncogene tyrosine-protein kinase SRC
SH2	SRC homology 2 domain
SH3	SRC homology 3 domain
SMC	sequential Monte-Carlo
SNV	single nucleotide variation
SNP	single nucleotide polymorphism
TKI	tyrosine kinase inhibitor
VEGFR	vascular endothelial growth factor receptor

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Chapter 1

Prelude

Chronic myeloid leukaemia (CML) is a moderately rare disorder, yet there is an unanticipated abundance of literature on the subject. Why is that the case? CML has no risk factors that we can realistically impact, occurs mainly in the elderly, and is rather slow acting. However, due to its singular cause, studies of CML have advanced our understanding of the molecular mechanisms of cancer and contributed in a grand way to the field of targeted therapies; treatments designed to act on the central driving impetus behind a malignancy.

In the late 90s, a breakthrough improvement to CML treatment regimens was discovered. An inhibitor targeting a unique oncoprotein present in every CML cell was discovered: imatinib. Survivability soared with over a tenfold improvement in eight-year survival rates; a truly successful example of cancer being treatable by acting on a unique molecular driver and mercifully sparing healthy cells. Unfortunately, all was not well. After years of treatment, a significant fraction of the patients tended to relapse, no longer responding to imatinib. The new cancer cells were found to carry minor changes in the drug target. Mutations that grant some cells treatment immunity in turn allow them outlive their other cancer-cell competitors and, over time, evolution inside the cancer creates an untreatable phenotype. Such evolving resistance has since shown up in many other instances of targeted therapy. The slow growth of CML gives ample time for evolution to happen.

Since then, several more targeted drugs for CML have become available, with varying degrees of potency against the different causes of resistance. Nevertheless, the problem of resistance remains. Inventing new drugs only provides a new goal for ever evolving cancer cells. Thus to some extent, clonal evolution is the real enemy. Without an actual cure, controlling the evolution of resistance means we can still achieve a high quality of life and excellent long-term survival. In this work, I hope to have taken some steps towards creating a knowledge base and tools which may allow for more optimal medicine use, so as to prevent resistance and improve survival.

Chapter 2

Background

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2.1 Cancer

Cancer is a significant element of the human condition, and with the human lifespan ever extending, it has only gotten more prominent. It is currently the second leading cause of death, in part because it is common, and in part because in a majority of cases it is very difficult to treat [1]. Cancer is defined by an abnormal proliferation of some cells, in such a way that they infiltrate surrounding

tissues causing damage. The abundance of cell types and mechanisms that can create a malignant phenotype makes cancer, as a whole, incredibly heterogeneous, meaning that, loosely speaking, no two patients are the same (which is why most general cancer cures can be dismissed off hand). Several common properties of cancer cells have been identified [2]. These are not necessarily requirements for something to be classified as cancer, rather, they are the elements that contribute towards creating the invasive, ever replicating and disruptive cell phenotype that we recognize as cancer. These common hallmarks are, in no particular order:

1. **Immortality** Whereas most human cells can only ever divide a small number of times, cancer cells have no such limit.

2. **Sustained proliferation** Cancer cells typically reproduce whenever they can.

3. **Genetic instability** A loss of genetic repair mechanisms, and an increased chemical stress leaves most cancers prone to accumulate further mutations.

4. **Resisting cell death and growth suppression** Ignoring the bodies signals to stop replicating enables malignant growth.

5. **Metastatis** Functioning cells typically stay in their designated body compartment, but cancerous cells tend to infiltrate other tissues.

6. **Avoiding the immune system** Some level of avoiding the immune system is required, as it could otherwise eliminate the malignant cells early on.

7. **Inducing angiogenesis** Creating new blood vessels helps maintain the energy supply required for rapid proliferation.

8. **Promoting inflammation** Inflammation alters the microenvironment in ways that may be conducive towards neoplastic growth.

9. **Deregulating energetics** Cancer cells are often powered mainly by glycolysis, even in the presence of oxygen. This may help produce chemical intermediates required for large scale synthesis necessitated by rapid proliferation.

This work is concerned mostly with chronic myeloid leukaemia (CML), a moderately uncommon variant of leukaemia with a characteristic slow progression. The topics discussed will often extend to Philadelphia chromosome positive (Ph+) variants of acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML)

and mixed-phenotype acute leukaemia (MPAL); but all of them are more complex conditions that typically do not respond as well to targeted therapy. CML incidence is around 0.6 to 1.8 cases per 100 000 persons, making up about 20 % of all leukaemias in adults [3, 4]. The other related leukaemias are all significantly more rare, with Ph+ ALL being the second most frequently seen among these diseases.

2.2 Precision medicine

With the advent of more accurate, faster, and cheaper (though not necessarily all at once) tests for a plethora of medically relevant factors, it is pertinent to ask: Can we use this patient-specific knowledge to improve treatment outcomes? In CML the most obvious application is in using next generation sequencing (NGS) data to choose the drug most likely to work against whichever mutated, resistant, version of tumour cells a patient may harbour. An accurate choice means treatment remains effective, whereas the wrong choice may mean higher than preferable drug doses are required, or in the worst case disease progression and eventually death. In other cancers which can often have a disparate set of molecular drivers, identifying unique details informs us what treatment options may be available, as there is no point in targeting a driver which is not present.

The ideas of precision medicine could be extended further. If a patient needs to take a break from treatment, when is it most appropriate? How should we prepare for such a break, and how should it best be followed up? To make optimal choices we need optimization tools, and to make such tools we require knowledge about the basic interactions that generate the disease. Ultimately, personalized treatments need not be perfected all at once. As our knowledge of the factors that affect cancer progression increases, gradual individualized improvements can be made in the cases where they are justified.

2.3 CML biology

CML is rare among cancers in the near omnipresence of a singular driver protein, and it was the first cancer discovered to have such a unique driver [5]. A chromosomal translocation between chromosome 9 and 22 forms the so-called Philadelphia chromosome (Ph) (Figure 2.1). It holds the constitutively active tyrosine kinase BCR-ABL1, resulting from a fusion of the proto oncogenes Abelson tyrosine kinase (ABL1) and breakpoint cluster region (BCR). The translocation which creates the Ph chromosome happens chiefly at random, with radiation exposure being the only known external contributor. In fact, the known risk factors for CML are only age, ionizing radiation exposure, and sex (with males being at a slightly greater risk). Several variants of BCR-ABL1 have been identified,

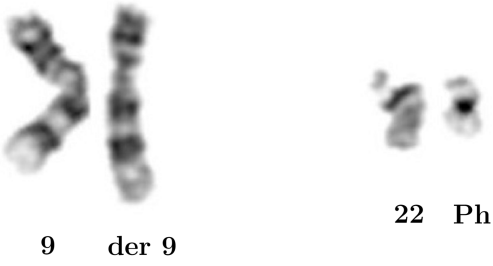

Figure 2.1: A partial karyogram of a Ph+ cell showing the result of the 9–22 chromosomal translocation: the Ph chromosome, and the derivative (der) chromosome 9 formed as a complement. Adapted from [7].

owing to different breakpoints in the translocation and different splicing of the resulting gene; the most common in CML is the 210 kDa variant (the $P210^{BCR\text{-}ABL1}$ isoform) [6]. The other isoforms tend to produce other leukaemias, such as Ph+ ALL. Given its status as a singular driver, and because it is only present in cancer cells, BCR-ABL1 is the primary target for CML treatments. There are disease states similar to CML without any variant of the Ph chromosome, but they should more appropriately be classified as different diseases (and they are very rare comparatively speaking).

On its own, ABL1 codes for a tyrosine kinase with mixed tasks in normal cell regulation. Tyrosine kinases in general are typically responsible for activating various cell processes by attaching a phosphate to a tyrosine of some targets. BCR is ubiquitously expressed with a range of functions, though none particularly vital as demonstrated by the overall very viable BCR–knockout mice. However, post fusion, BCR-ABL1 becomes an incredibly prolific enzyme. The SRC homology 3 domain (SH3) typically responsible for regulating the activity of ABL1 is stripped away (among other things), rendering BCR-ABL1 able to freely phosphorylate a number of substrates, including itself. BCR-ABL1 has three main effects causing the oncogenic transition: Mitogen activation, apoptosis resistance and reduced adhesion (all typical hallmarks of cancer). Possibly more than one mitogen-activated protein kinase (MAPK) is triggered by BCR-ABL1, directly or through binding to the autophosphorylated form [8]. From the initial lesion and Ph–chromosome formation it takes about 5 to 10 years until noticeable symptoms start to appear [9] (see Table 2.1 on page 28).

There is some contention as to whether the presence of the Ph chromosome and BCR-ABL1 alone is sufficient to produce CML. Notably, the Ph chromosome can sometimes be detected in healthy people (who also do not acquire leukaemia later

on). This may be because it did not occur in the right type of haematopoietic stem cell (HSC), or perhaps because immune response, or random chance associated with the growth and death of cells, eliminated the cancer stem cells (CSCs). The age-incidence of CML indicates that BCR-ABL1 is the singular cause [10]. It has also been shown that BCR-ABL1 alone can create CML–like conditions in mice [11]. Thus, it is typically believed that no oncogenic adaptations beyond the Philadelphia chromosome and BCR-ABL1 are required to cause CML if the Ph–chromosome occurs under the right circumstances.

2.3.1 Cancer stem cells

Cancer stem cells as a concept was originally discovered by noting that the colony-forming potential of cancer cells was generally rather low, with only a comparatively small fraction of all cells having any significant replicative ability [12]. It seems that in certain cancers, only a fraction of cells are responsible for sustained replication. Note how this is different from a situation where replication is unevenly distributed, but all cells have the ability to do so if given the right circumstances (which for instance can happen with regards to surface/core structures of a solid tumour). The ability to identify cells of a distinct phenotype and demonstrate their colony-forming ability in iterated experiments is the defining characteristic of CSCs [13].

CML follows a CSC model, almost to a tee. Similarly, Ph+ ALL also has a CSC structure [14]. In leukaemias it is possible to identify stem cells, i.e. cells with colony forming ability (using biochemical markers) [15]. Primordial cancer cells form a stable or very slowly growing colony in the bone marrow, emulating to a large degree normal haematopoiesis [16]. These primordial cells, or CSCs, produce in turn a hierarchy of increasingly differentiated cells, with increasingly higher growth rates and lower degrees of self-replication. Unlike in normal haematopoiesis, aberrant differentiation in CML ultimately results in non-functional cells. The exact structure of CSC replication and differentiation remains to be elucidated, though the cells are commonly grouped into four groups: CSCs, committed progenitors, differentiated cells and terminally differentiated cells, with the first two groups undergoing significant amounts of self-renewal [17]. Consequentially, if a resistance mutation was to happen, it could only ever last if it occurred within the former two, since without self-renewal, the mutant heritage is doomed. It is possible, that the structure should be more fine-grained with more than four cell compartments, though the exact structure is unknown. The compartment structure interplays with the acquisition of mutations, since smaller groups of self-renewing cells also means fewer persistent mutations in practice. In normal haematopoiesis, the strict hierarchy very effectively suppresses mutations from taking hold [18]. CML likely has a somewhat more disrupted hierarchy allowing a larger pool of cells to acquire and sustain mutation development. That would more adequately explain the high frequency of mutants. It has

been suggested that the CSC pool in CML is about 100000 to 500000 cells at diagnosis [17,19]; although, the biomarker used to identify CSCs may hide deeper hierarchies [15]. It is possible that only a handful of true stem cells account for that population [20]; though this does not satisfyingly account for the frequency of persistent resistance mutations.

Tyrosine kinase inhibitors (TKIs), the revolutionary group of targeted therapies discussed later on, can slow the growth of, but not kill, CSCs [21–23]. This has implications for the modelling of evolution resistance in CML, as the comparative fitness of mutant CSCs is altered, but the treatment cannot cure.

2.3.2 In the clinic

CML is typically divided into three stages: Starting in the slow growing and relatively harmless chronic phase (CP) it eventually transitions through the accelerated phase (AP) to the blastic phase (BP), also known as blast crisis (BC) [24]. A majority of patients are diagnosed in the CP, which is characterized by an increased presence in the peripheral blood (PB) of white blood cells carrying BCR-ABL1, albeit without major harm. CP CML is often asymptomatic, or has very mild symptoms (Table 2.1 on page 28). In the early stages, it is most likely that the disease is detected by coincidence, such as on a blood test. Treatments starting in the CP, the earlier the better, have excellent prospects, and the tumour can typically be suppressed to the point where BCR-ABL1 cannot even be detected using sensitive molecular methods. All treatments fare better in the CP than in the later stages. However, even with good treatment response, some residual disease tends to remain; it is believed that CML CSCs cannot be killed by available treatments. Thus, the CSCs pool continuously grows hidden in the background [17]. With or without treatment, a portion of all patients eventually transition to the AP, although it is often heralded by some resistance adaptation. The AP is distinguished by some inherent treatment insensitivity, and a greatly increased amount of final dysfunctional BCR-ABL1 carrying cells. At this stage, symptoms are also often more prominent. Finally, BC is typically unresponsive to treatment, grows extremely fast and generally carries several additional oncogenic adaptations beyond the Ph chromosome. BC is characterized by a greater presence of immature blasts in the PB than what can be detected during the AP. At this stage, survival outlook is bad and symptoms are severe.

Preventing the progression from CP to BC is the main goal of CML treatments. While a cure would be preferable, it is possible to live a long and high-quality life in the CP. The transition to BC typically comes with a series of additional genetic lesions [8]; it seems that BCR-ABL1 increases mutation rate in general. As a consequence, additional oncogenic pathways render BC phase disease much less sensitive to drug treatment. Probably, these genetic changes are required for the transition to BC in the first case [24]. It may be that the genetic

lesions characteristic of BP happen already in the AP, and that the distinction is functionally less significant than the transition from CP to the later stages [25].

Ph+ ALL has much in common with BP–CML, and is similarly difficult to treat. Our modelling effort will focus mainly on the CP since so long as the treatment can remain efficacious, the CP can last forever with no major harm. This in turn means that any results are first and foremost applicable to CML.

Diagnostically, CML patients are often placed in to risk groups according to their Sokal or ELTS score; both of which take factors such as age, blood counts and spleen size into account [26,27]. These measures offer a reasonably accurate prognosis. Treatment response is divided into three categories: Haematological response, detailing whether there are abnormal blood counts, cytogenic response, representing whether there are Ph+ cells in the bone marrow, and molecular response, which is a measure of whether BCR-ABL1 can be detected using PCR. Achieving major molecular response (MMR), a 1000–fold (or greater) reduction in BCR-ABL1 on a PCR–test, is associated with a very good long-term prognosis [27].

2.4 Treatment options and tyrosine kinase inhibitors

Historically interferon alfa (IFN–α), often with cytarbine, were used to treat CML with moderate success [30]. Before that treatments consisted of busulfan and hydroxyurea [31]. IFN–α is a drug derived from human blood that replicates some immune functions, whereas the other three are different forms of cytotoxic chemotherapy. Since the early 21st century, targeted treatment in the form of tyrosine kinase inhibitors (TKIs) has become the dominant treatment modality. TKIs offer excellent effect with rather limited side effects. Several BCR-ABL1 TKIs are available now, which opens the possibility of selecting the best drug for a particular patient and situation. That selection offers both a partial solution to providing effective treatment in all cases, and a new problem entirely in making an accurate selection (see Table 2.2 on page 29). Another option involves an allogenic haematopoietic stem cell transplant (allo-HSCT); practically speaking replacing a patients bone marrow, including any CSCs therein, with that of a donor. The difficulty of finding compatible matches, and the rather pressing dangers involved, renders this a less common option. Unlike TKIs, it does however have the capability of curing CML entirely. This work will focus on targeted therapy options however, as any issues pertaining to allo-HSCT are entirely different.

Nowadays, CML treatment typically involves starting with imatinib, or more and more commonly a second generation TKI. Lack of response or resistance to imatinib is typically followed by switching to a second generation inhibitor, although some mutations may respond to higher doses of imatinib [32]. A lack of response to a second generation TKI should be followed by sequencing for mutations, preferably with NGS, as it is possible to try and select another second

generation TKI that may work. If the mutation T315I[1] is discovered, ponatinib should be used. At this point, it's also advisable to search for allo-HSCT donors [29,33], as progression to AP is likely.

In Ph+ ALL, treatments typically consist of a combination of chemotherapy with TKIs. Treatment is often successful initially, but long term prognosis for ALL with such a protocol is not as good as in CML. The initial response is thus used to set the stage for allo-HSCT, which can be curative [34].

Imatinib

Imatinib was the first TKI to be approved, and constituted an impressive improvement over the old CML treatments [35]. More than a tenfold improvement in eight-year survival rates has been reported [31]. Despite this success, about a third of all patients had to discontinue imatinib treatment for some reason (e.g. intolerance, lack of response) after a median followup of 76 months [36]. Imatinib is now available as a generic formulation, which makes it the cheapest treatment option by far. It performs similarly to the branded version, and as imatinib is a potent treatment, despite successors, first line imatinib treatment is currently the most cost effective option [27,29].

Imatinib, and most BCR-ABL1 TKIs are ATP–competitive inhibitors that attach reversibly to the ATP binding site with the protein in the inactive conformation [37].

Second generation TKIs

Treatment failures with imatinib, and the possibility of producing more potent and specific drugs led to continued developments. Three second generation drugs are available: dasatinib, nilotinib and bosutinib. Dasatinib and nilotinib are both significantly more potent than imatinib in vitro. The general trend is that the second generation TKIs produce a stronger response faster than imatinib. An improved long-term survival has not been unequivocably established, but evidence does point to a more sustained response [29]. What is fairly certain however, is that these drugs are less vulnerable to drug resistance, especially resistance mutations [38]. The ability of dasatinib and bosutinib to also inhibit proto-oncogene tyrosine-protein kinase SRC may be important in shutting down certain drug resistance pathways.

Unlike the other drugs, dasatinib is a so-called type I inhibitor, meaning it binds to the active conformation of BCR-ABL1. As the active conformations of all tyrosine kinases are more similar than their respective inactive conformations, this may contribute to additional off-target activity. However, the lowered specificity can also protect against resistance mutations [37].

[1] An alphanumeric string like this tells us what amino acid residue has been replaced and its replacement. In this instance, a threonine (T) at position 315 in the protein, has been replaced by an isoleucine (I).

Using second generation TKIs as first line treatment is becoming more and more common. They have different side effect profiles, which is valuable in adapting to individual patients. However, the second generation drugs are associated with rare but severe side effects and their overall impact on quality of life have not yet been sufficiently evaluated [39].

Ponatinib

Ponatinib was developed to deal with one specific failure mode that all previous TKIs have in common: the so-called gatekeeper mutation T315I, which occurs in the drug target BCR-ABL1 [40]. In fact, no single nucleotide variation (SNV) provides significant resistance towards ponatinib, but there are compound mutations that do [41]. Its success is hampered somewhat by exceedingly severe side effects, with thrombotic events, in particular, being common, limiting its use. This is possibly caused by it also being a vascular endothelial growth factor receptor (VEGFR) inhibitor. As such, it is only used when the T315I mutation has been confirmed [42].

Experimental drugs

Development is still ongoing, with several experimental drug candidates available. One of the most interesting and significant is asciminib. Asciminib is the first real success in a long line of efforts to develop an inhibitor targeting the myristoyl pocket of BCR-ABL1 [43]. By binding to a different site, it is no longer vulnerable to many of the causes of resistance that occur in regular ATP–pocket TKIs. It also enables dual targeting for the first time, and appears very promising in combination therapies (Paper III). Combinations with nilotinib or ponatinib both seem to be promising options [43–45]. Previous efforts (GNF–2, GNF–5, etc) have struggled with reaching effective potency [46].

Axitinib is another drug of interest, as it is effective against the highly problematic T315I mutation, but not against unmutated CML cells. It is already approved for use in renal cell carcinoma (RCC), thus its safety is already established. However, whether it is actually useful for treating CML is not yet known. In Paper I we also touch upon rebastinib, a proposed TKI which is no longer studied, but which was included because there was reliable data available for it.

Theory suggests that to cure CML it is necessary to eliminate the CSCs [47]. An option is to combine TKIs with drugs intended to kill CSCs specifically. It has been shown that TKIs can slow the growth of, but not eliminate CSCs, so, an additional drug targeting CSCs may allow for an actual cure [48]. It is also possible to use combinations of TKI with other methods, for instance, nilotinib and radiation therapy may be benificial in Ph+ ALL [49].

2.5 Drug resistance

As I have touched upon several times, a major issue in CML treatment (and in many drug-based cancer treatments), is drug resistance. Resistance against surgery or radiation is typically of less concern; surgeons have yet to require self defence training to combat particularly aggressive cancers. Jokes aside, drug resistance is a major issue responsible for most CML deaths [5]; indeed, drug resistance is observed for most targeted cancer treatments. Reports of imatinib resistance started to roll in while it was still in trials [51], and similar accounts exist for subsequent drugs. Resistance adaptations are categorized into two groups, BCR-ABL1 dependent mechanisms, such as mutations and increased expression, or BCR-ABL1 independent mechanisms, such as drug transporter upregulation and alternate pathway activation [37,52].

2.5.1 Resistance mutations

There are many observed mechanisms of resistance in CML but most prominent and numerous are mutations in the kinase domain (KD) of BCR-ABL1, with well over 40 different mutations suspected to confer resistance towards imatinib [53]. KD mutations act in several ways to lower the binding of drugs. Mutations near the binding site, like T315I typically interfere directly with binding through steric clashes or removing hydrogen bond targets [54], but distal mutations can change the dynamics of the protein in such a way that it lowers the probability that a drug can bind [55]. A small amount of lowered efficiency is tolerable, and the second and third generation drugs seem to have a greater overhead, in the sense that achievable plasma levels are high enough relative to its effective range that weakly resistant mutants can be suppressed to such a degree that the treatment remains functional. Some mutations also increase the activity of BCR-ABL1, which could also create resistance independent of affecting drug binding [54]. KD mutations are slightly more common in patients that become resistant to imatinib as a first line therapy than they are in patients that become resistant after using a second generation TKI as a first line therapy [56].

The bulk of TKI mutation studies are done in Ba/F3 cells, known to be interleukin 3 (IL-3) dependent in their natural state. However, when transfected with some oncogenene, they can be made IL-3 independent so long as the oncoprotein remains active [57]. This has made it extremely popular as an in vitro scaffold for testing kinase inhibitors, as well as the effects of KD mutations [57], since it is significantly easier to manufacture a variety of Ba/F3 mutants than it is to produce a similar library from a real cancer cell line. Since BCR-ABL1 is such a singular driver, its fitness in Ba/F3 is believed to correlate decently with the fitness of real CML cells carrying the same mutation, which is corroborated by clinical data [58].

It is not entirely known whether mutations are present in CML CSCs before drug treatment starts, or whether they occur during treatment. Preexisting mutations can sometimes be detected in patients, and those mutations tend to cause resistance later on [59]. In practice it seems likely that both preexisting and acquired mutations occur, but it is possible that one is more common than the other. The argument for preexisting mutations largely comes down to small fitness differences between mutated and unmutated cancer cells without the presence of drugs. Thus, if a mutant occurs in the early phase when the CSC pool is expanding from a single initial cell, it is unlikely to be randomly eliminated, and probably expands as well, into a pre-treatment population. Significant expansion between initiation and detection means a great number of opportunities for mutations. The argument for acquired mutations is in large part that mutations in practice can occur after very significant delays; so long that it seems unlikely a dormant mutation could have been present for years. Such cases could however be accounted for by random delays from stochastic effects while there are still very few mutations [60]. NGS is not guaranteed to detect mutations at the start of treatment [61]. Either because NGS methods still are not sensitive enough to detect mutations in very small subpopulations of cancer cells, or because there is nothing there to detect. Once a mutation is present, it will expand relative to the unmutated population through clonal evolution so long as treatment is present, which imparts a significant growth advantage to resistant cells. Modelling predicts that preexisting mutations should the most common [62], however in CML in particular, the long term drug exposure and overall treatment insensitivity of the critical CSC–pool provides the perfect conditions for resistance mutations that emerge later during treatment.

With the advent of multiple drugs targeting BCR-ABL1 all KD single nucleotide variations (SNVs) have at least one drug that will inhibit them [41]. The different TKIs do not necessarily share their resistance mutations (Figure 2.2). The most infamous mutation is T315I, aka the gatekeeper mutation due to its position at the mouth of the ATP binding pocket. After the discovery of ponatinib it has become treatable, but ponatinib is also comparatively dangerous which may hinder its application. Other experimental drugs may soon provide options however.

There are also double mutants against which no drug (barring possibly asciminib, it is still unknown) are effective [41]. Much in the same way that one mutation can be acquired, nothing stops those cells from mutating in turn, thus, one is always left chasing a moving target. Compound mutations are when two ore more SNVs occur in one copy of BCR-ABL1. Different mutations occurring on different copies of the gene (often in separate cells) in one patient, is also known to happen. Either situation may provide multi drug resistance, and both have been observed clinically. However, they are not a common cause of resistance towards ponatinib [61], despite such mutations being known

to exist [41]. Compound mutations occur especially in cases where a patient fails multiple sequential treatments [56] and this type of sequential resistance acquisition is readily observed in in vitro experiments [63].

Finally, while mutations may seem insurmountable, these changes to BCR-ABL1, a critical cancer component, are not without constraint. A mutation is not viable if it damages kinase activity beyond repair, as ultimately the kinase activity is what drives a Ph+ malignancy. The known mutations all seem to maintain a viable kinase [56, 64]. It is theoretically possible that a series of compound mutations could restore function despite individually being deleterious, but the near infinitesimal odds of acquiring such a set of mutations simultaneously means it is not of any clinical significance. Indeed, it seems more likely that compound mutations are inherently constrained by diverging too far from the original KD sequence and losing functionality, as they are not overly common in patients [61]. No reported CML resistance mutation could be found occurring naturally when cross referencing COSMIC and the ExAC project [53, 65] (aside from a likely misidentification, see Summary and Discussion). Thus it does not seem likely that one would inherit germline TKI resistance in CML. Resistance mutation single nucleotide polymorphisms (SNPs) did occur in some other targeted therapies and cancers.

2.5.2 BCR-ABL1 overexpression

BCR-ABL1 overexpression works by increasing the production of the drug target thus maintaining some effect as TKI concentrations no longer effectively inhibit all of the kinase activity. It is not a very common resistance mechanism, but has been observed [67]. However, it is frequently observed in cell lines, and seems connected to the emergence of mutations [68], possibly through increased reactive oxygen species (ROS) [69].

2.5.3 Altered drug transport

As the intracellular presence of TKI is required for them to take effect, changes in drug transport that lower intracellular concentrations can cause drug resistance. Drug transporter upregulation (mainly of organic cation transporter 1 (OCT-1), P-glycoprotein 1 (ABCB1) or ATP-binding cassette super-family G member 2 (ABCG2)) can lower the intracellular concentrations of inhibitors thus restoring signalling. There are reports showing synergistic effects of transporter inhibitors in vitro [70, 71]. Notably however, the third generation TKI ponatinib is an ABCG2 inhibitor [72]. Ponatinib is also not a drug transporter substrate in the first place [73], meaning it should be immune to this mechanism. Imatinib influx seems dependent on OCT-1, and thus lowered activity or downregulation is associated with poor response or resistance to imatinib [71].

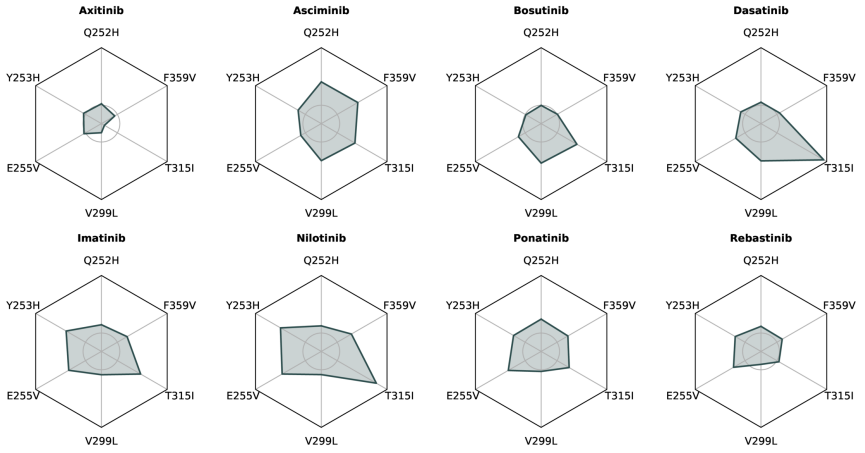

Figure 2.2: SNVs in the BCR-ABL1 KD convey different degrees of resistance towards different TKIs [66]. Depicted: base 10 logarithm of the mutant 50% inhibitory concentration (IC_{50}) and the non mutated IC_{50} ratio for some select mutations and drugs. The central grey circle indicates an equal ratio, i.e. no selective advantage. A large value (i.e. far from centre) for a mutation indicates that it is favoured by selection in the presence of that drug. Note that achievable drug concentrations, and hence what mutations can be considered treatable varies between the drugs, is not an element of this figure. A favourable mutation may still be treatable, as well as the opposite.

2.5.4 Alternate pathway activation

BCR-ABL1 is the canonical driver in CML, but it is possible for other enzymes to take over its role. Most prominently, the activation of proto-oncogene tyrosine-protein kinase SRC (SRC) family kinases and aurora kinases [52,74]. Part of the success of second generation inhibitors may be attributed to off target kinase activity preventing alternate pathways from restoring signalling [75]. Activation of more oncogenic pathways is also a hallmark of BC, which in itself tends to respond much worse to drugs [24].

2.6 Clonal evolution in cancer

Evolution works on a base principle of reproduction with variation, and cancer provides all the necessary elements. Cancer proliferates by cells making copies of themselves; imperfect copies that only approximate the function of their progenitor. The variation comes in many forms, cancer is almost universally defined by a degree of genetic instability from a loss of gene repair function and

an increased chemical stress causing more genetic damage [76]. The emergent population dynamics provides the framework in which evolution happens [77].

Variation alone can drive evolution. Over large timescales, genetic drift where genes are randomly replaced is the most important driver of evolution. But under the right conditions, selective pressure is a much more rapid actor. Selection, and Darwinian evolution, happens when the environment stratifies the existing variation in a population into phenotypes more and less likely to survive [78]. Individuals (cells) more likely to produce a surviving lineage are said to have a higher fitness than the rest. Depending on the specifics of the system under study, fitness could be defined in many forms. In cancer, often times the growth rate of cells are a good proxy for their eventual fitness [77].

If we narrow our view to a small set of properties, a population often consists of only a single group; while there is likely some variation (genetic or otherwise), it may not be present in any element of concern. Suppose further that a new individual shows up, functionally different in something we care about. Initially, whether those traits remain or are lost is very random. A single individual is greatly affected by random death, or a random string of successful reproductions. If its traits provide no major advantage, i.e. it is *neutral*, and has no fitness advantage or disadvantage compared to the rest of the population, this randomness persists, and rarely it may eventually come to take over the population [78]. If it is detrimental, the likelihood that it is eliminated increases, but it may still be very slow to get rid of the few last cells, as randomness dominates in that domain. On the other hand, if it has a high fitness, it is expected to gradually become a greater part of the population. As it becomes more numerous, its progress becomes less and less random; with a fitness advantage and a good deal of the total population, it is likely to take over and become *fixed*. The final stages may be slow and unpredictable however, for the same reason that lower fitness cells are not always effectively completely eliminated.

Mutations come in several forms, but, given the defining and critical nature of an organism's genome, not all changes can be tolerated. Aside from all important regulatory functions, the primary task of DNA is to encode proteins. Triplets of nucleotides, codons, translate into amino acids in a code shared very closely between all forms of life. The most common and simple form of mutation is a SNV, i.e. a single nucleotide in the DNA is changed for another. Notably, such a change does not allow for any amino acid to be changed into any other. In fact, due to redundancies in the genetic code, in most cases much fewer than the maximum nine options are available. In addition, not every nucleotide change is equally likely; there is a distinction between so-called transitions and transversions [78]. This is due to some nucleotides being structurally similar, and those replacements are more likely to happen. Taken together, SNVs are deceptively complex, especially so on a functional level where the amino acid

sequence is what actually matters. This variability predicts in part the frequencies of resistance mutations observed clinically [79].

2.6.1 Evolution of resistance

In the case of cancer drug resistance, there are a few things to keep in mind. Drug treatment designed to kill cancer cells (specifically or by targeting rapidly dividing cells in general) by their very nature stratifies the cancer cells by their ability to tolerate the drug. As fitness becomes strongly determined by drug tolerance, more and more of the cancer cell population is expected to come from such a lineage. Note how this can become an iterative process, as drug tolerant cells reproducing with variation can turn into highly tolerant cells which may produce resistant cells and so on as selection acts continuously. It can also happen in single large steps, if unique mutations provide massive fitness advantages (as is the case in CML).

One important addition to this is the CSC concept discussed before. If only CSCs sustain the overall replication, then, evolution can only happen in CSCs. Unless, of course, random changes happen to grant a differentiated cell CSC–like abilities, which would be extremely favourable for its lineage. Ignoring that unlikely exception, this means we can focus on the CSC pool in malignancies where such a model is appropriate.

Finally, while cancer is highly heterogeneous, resistance evolution still often starts in the same place. As a non-contagious phenomenon, genetics that are conserved in the human population overall, are similarly conserved in the initial cancer cell. For Ph+ cancers, there is no relevant variation in BCR-ABL1, thus practically all patients have the same starting point for the evolution of BCR-ABL1 KD resistance mutations. In a simplified world, this means the same strategies could be applied to all. In practice however, even the very uniform CML is more heterogeneous than that. Nevertheless, starting from the same template makes the evolution of resistance in cancer a similar but simpler phenomenon that preventing antibiotic resistance or similar. In cancer, each patient presents a new opportunity (and challenge), in stark contrast to contagious disease where evolutionary adaptation can be propagated to new patients.

2.7 Why model cancers?

Naturally, it is not possible to casually experiment on very sick individuals, and any and all forms of cancer need to be considered a serious threat. For this reason, medical experiments go through a series of steps, typically starting from mechanistic studies of biochemistry, going through innumerable in vitro experiments on cells before even animal testing is justified. All that before human experiments are considered. As such, only an extreme minority of all possible

ideas could ever be tried in practice, in part because testing is so limited, but also because the space of possible treatment actions is unfathomably large. Using mathematical and computer models, we can consider and select from a much wider base of possible final experiments. Options that otherwise would not have been considered can be highlighted and further examined [80].

Secondly, modelling is an element of explaining observations. Multiscale models can now explain the detailed action of many drugs [81]. A deeper understanding of internal mechanisms can spark new inventions. For instance, studies of biological signalling networks have led to discovering previously unknown druggable mechanisms [82]. An improved understanding of protein kinases, which frequently act as potent oncogenic drivers, also helps in guiding the development of new treatments [83]. Modelling has also helped explain previous failures in using angiogenesis inhibitor therapy [84].

2.8 Aims and approaches of cancer modelling

There are many ways to approach either of the above two goals, i.e. creating a framework within which to run in silico clinical trials, or learning new theoretical aspects of cancer that may prove useful. CML makes an excellent model because it is relatively simple. This largely comes down to the characteristic single driver, which vastly cuts down on the variations possible [85]. The initial evolution of resistance is thus simplified, although progression towards BC brings back much of the complexity (and difficulty in treating). Other forms of cancer often require more complex models to account for spatial structure, rapid growth and invasion, etc.. I will make a very sweeping distinction into groups of (I) deterministic and continuum models and (II) stochastic and individual based models. As a rule, models of type (I) are often easier to analyse, as they decompose typically into differential equations.

Before discussing specific models, it is worth dwelling briefly on cancer growth. The deregulated growth of cancer cells is to a large extent dependent on nutrient availability [77]. Having access to practically unlimited nutrients produces the familiar exponential growth, i.e. if every cancer cell divides once per day, starting at 1 cell we get 2, 4, 8, 16, 32, ... cells. More formally, for a growth rate r starting with N_0 cells we have

$$N(t) = N_0 e^{rt}. \tag{2.1}$$

This quickly becomes absurd. In the above example, we go from 1 to over 10^9 cells in one month, and reach 10^{18} cells one month after that (which is obviously impossible[2]). That said, many models of early cancer progression are based around exponential growth, as early on the cancer cells are few and the human body's resources are plentiful.

[2] 10^{18} human cells would form a sphere over 16 m across weighing more than 2 000 000 kg.

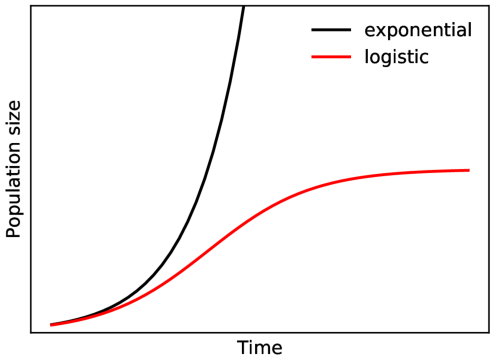

Figure 2.3: Exponential and logistic growth are initially similar but quickly diverge completely.

A more stable, and very popular model is logistic growth, where the population size over time follows the logistic function

$$N(t) = \frac{K}{1 + \left(\frac{K-N_0}{N_0}\right) e^{-rt}} \tag{2.2}$$

often written as the differential equation

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right). \tag{2.3}$$

K above is known as the carrying capacity, i.e. the equilibrium population size. In Equation 2.3 we can see that logistic growth is consistent with a pairwise death rate for all cells [86], further elaborated on in the Methods chapter. This very effectively limits the population size, see Figure 2.3. For a non-solid tumour, like CML, this is generally a reasonable model, and one I will often adapt in this thesis. In fact, CML is commonly modelled as not growing at all. By letting cell birth and death occur in perfect lockstep, a model can be made which merely sustains pool of CSCs. Despite being a somewhat unrealistic approach it can provide valuable insights and is qualitatively similar to having logistic growth at its end-point equilibrium.

For completeness, I should briefly discuss solid tumours. In a solid tumour, nutrients need to somehow reach all the (living) cells, but can only get there through a limited surface area [87]. This is why vascularization, or angiogenesis, is so critical to the progression of many cancers [88]. As a consequence, there are often area and volume dependent terms [89]. Since nutrients cannot reach the centre, solid tumours generally have a necrotic core, with only the regions near the surface actually growing. Leukaemias do induce angiogenesis, but there is no

strong evidence that it critical. As such, we can remain reasonably confident in considering leukaemias to be well mixed, at least from a nutrient perspective [90].

2.8.1 Treatment effects

The effects of treatment are modelled in disparate ways [91]. We need to define the idea of a dose response relationship. The most common metric is the IC_{50}, i.e. the concentration of a drug required to reach 50 % inhibition. It is important to keep in mind that the exact metric of inhibition used may influence the value and its interpretation. TKI are often evaluated by measuring their effect on the growth of Ba/F3 cells. The relationship between drug concentration and effect can, in many cases, be adequately described by the equation

$$f_v = \frac{1}{1 + \left(\frac{[I]}{IC_{50}}\right)^m} \tag{2.4}$$

describing how the fractional velocity f_v, i.e. the amount of activity (for instance growth, as above), relates to the inhibitor concentration $[I]$. The so-called hill coefficient m describes the cooperativity, essentially a higher m indicates that additional inhibition is more and more effective, and vice versa. Note how Equation 2.4, suitably rearranged, forms a logistic function, as follows.

$$f_v = \frac{1}{1 + e^{m(\ln[I] - \ln IC_{50})}} \tag{2.5}$$

This is often exploited to infer m and the IC_{50} from experiments by fitting a logistic function to the data (Figure 2.4). With these parameters known, it is possible to predict the effect given a certain drug concentration, or conversely, to select the drug concentration that would produce a desired effect. These properties makes the IC_{50} useful, both for predictive modelling, but also as a clinical tool, and many authors have investigated the relationship between different KD mutations and the IC_{50} (e.g. [38, 92]).

Even so, the effects of modelling can take many forms. The fractional kill hypothesis states that a treatment period kills some fraction of all cells; the size of which could be determined using Equation 2.4. Assuming that treatments directly kill cells may be appropriate for some approaches. For instance, radiation is often modelled as directly killing a number of cells according to the linear-quadratic model [49, 93]. Alternatively, treatment effects could be modelled by assuming drugs interact with the cancer growth rate. Changes to the net growth rate can be further distinguished into those affecting frequency of cell division, and those increasing apoptosis. Naturally, a single treatment may fall into any or all of these categories. In CML, treatment effects are most commonly seen as reducing the frequency of cell divisions [17, 20], since we know TKIs do not kill CML CSCs. Because of how the CML CSCs reproduce in an amplifying cascade, even small

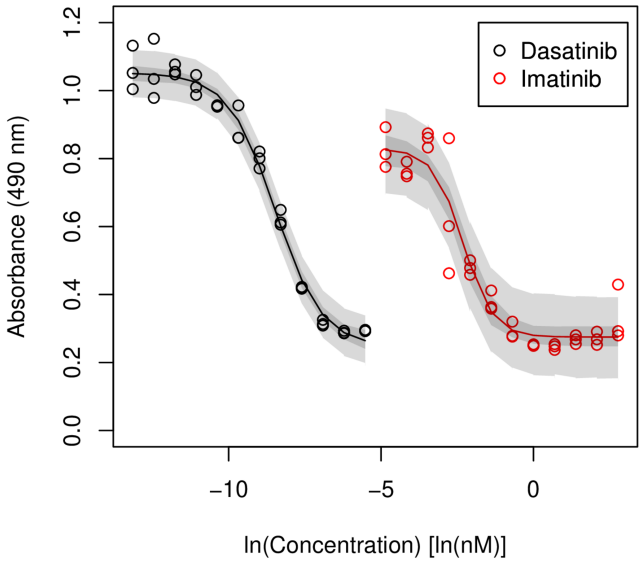

Figure 2.4: Results of logistic curve fitting to dose–response experiments for two different TKIs in KCL-22 cells. The IC_{50} is the given by the steepest point of the slope, and m defines the curvature around the inflection point. The absorbance in this experiment is directly proportional to the number of living cells. The dark grey regions show the 89 % compatibility interval for the mean, and the light grey regions show the 89 % compatibility interval for posterior predictive simulations.

changes to growth rates can greatly affect the final number of cancer cells. A tenfold decrease in CSC growth rates can reduce the number of differentiated CML cells in PB by over a thousand-fold [17].

2.8.2 Evolution

Cancer cells divide and produce genetic variation[3] with no aim or direction in mind. Selective forces acting on the population let high-fitness clones expand, whereas low-fitness clones are eliminated by competition. Be that as it may, the forces of natural selection are not so precise in their effect. Stochastic noise greatly influences what cells eventually survive; even an exceptionally fit cell may not

[3] Although for evolution to work, variation needs merely to be inheritable, not necessarily genetic in origin.

outlive its competitors and models need to take this into account [78,94]. Growth and death discussed before form the scaffold upon which clonal evolution is built.

There are a myriad of modelling approaches for investigating evolution [85]. There are some recurring themes however; core structures from which derivative models originate. Some common approaches include the Moran model, a birth-death process, a branching process, cellular automata (Figure 2.5) and detailed biophysical/biochemical simulations. The approaches taken in this work fall mainly into the first three categories. For studying evolution, agent based models are often used which, in the context of clonal evolution in cancer, considers the cancer in terms of its cells. At a core assumption level, each cell is an individual that may do things like replicate with variation or die, although they can be grouped by similarity for purposes of analysis. In contrast to this type of models, things like continuum models or pharmacokinetics models (Paper III) are not concerned with the actions of individual cells. Continuum models are typically less suited for situations where some important group of cells exist in a very small quantity, such as the early history of mutant cells, but are very suited for modelling both large populations of identical cells and the microenvironment, in both cases because random effects are small and can be neglected without a major loss of accuracy.

In any type of model that includes mutations, it is very common to apply the *infinite site assumption*. It states that there is an infinite number of sites in the genome that can be mutated, and each mutation is in a new location[4] [95]. Hence, the infinite site assumption disallows backwards mutations where, for instance, a resistance mutation is lost through mutating back to the normal sequence. This often simplifies the mathematical analysis of models, but when performing numerical simulations it is typically not necessary and has no real impact.

2.8.2.1 Agent based models

The Moran model considers a fixed number of cells N. With each iteration one cell, randomly selected albeit weighted by the fitness of the cells, replaces one other completely randomly selected cell with a copy of itself [96] (Figure 2.5A). This has very well known solutions for the fixation probability (Figure 2.6), and is straightforward to simulate. A Moran process has no concept of time however, and the perfectly constant population size is somewhat artificial. One interesting application of the Moran model with two and three types is present in Werner et al. (2011) [97], where the adaptation of Ba/F3 cells towards imatinib is best explained by having the existence of some resistance adaptation that is likely but offers middling resistance and a rare but highly resistant mutant cell type. Another very clever extension involves embedding a Moran process in a

[4]With an infinite number of sites, a second mutation will *almost never* occur in the same position as the first, i.e. it can happen but has probability zero.

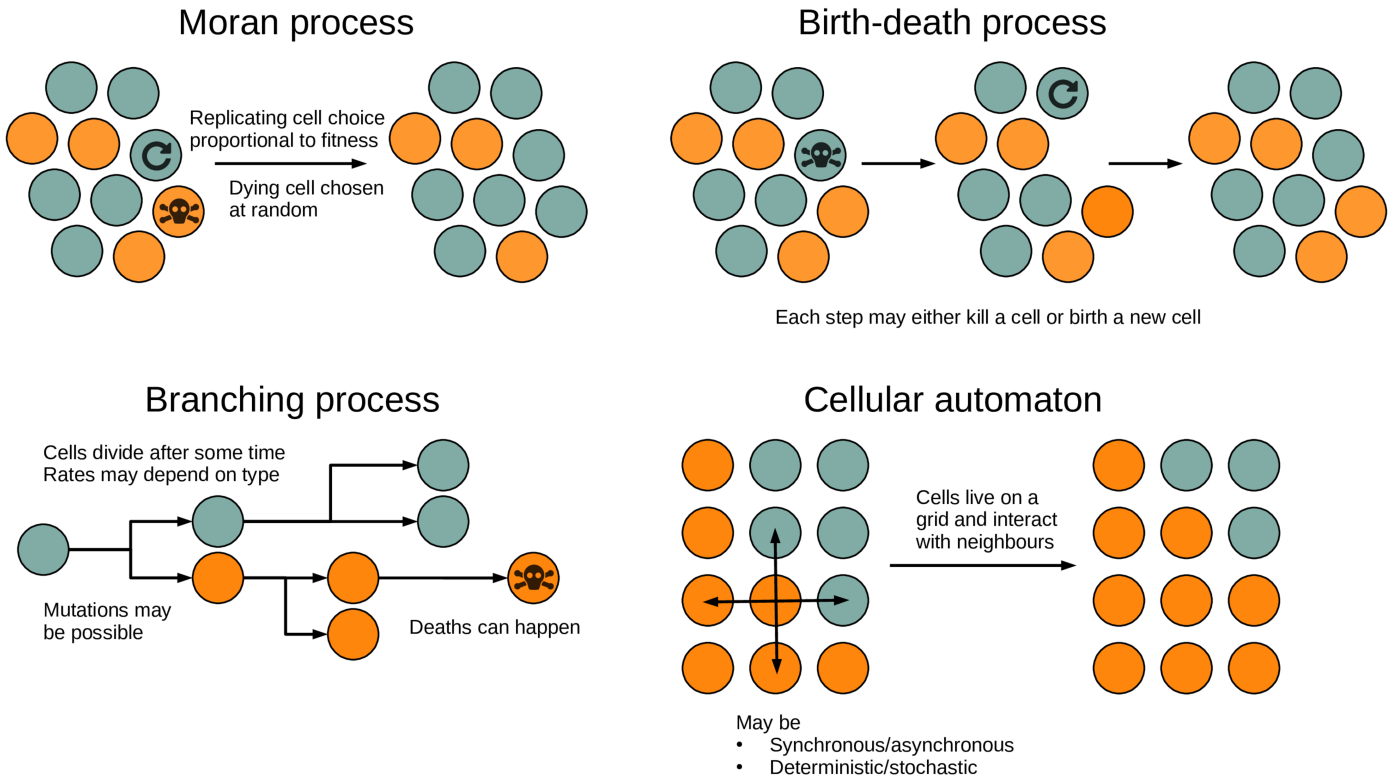

Figure 2.5: Different individual-based models for studying evolution. Innumerable variations are possible within any of these frameworks, and many other main structures are possible.

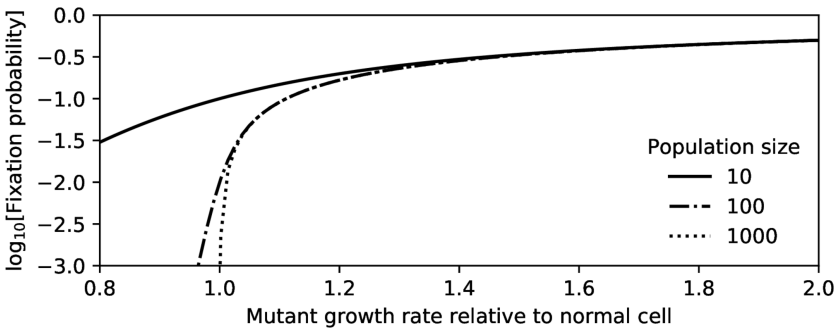

Figure 2.6: The fixation probability in a Moran process is exactly $1/N$ for neutral mutations, grows slowly for an increasingly advantageous mutation, and drops sharply for deleterious mutations. Many other models of evolution are qualitatively similar.

digraph, i.e. a graph where the edges have directions. Cells can only replace neighbours in the graph direction, thus graph structure influences the model. On a fully connected graph, this is identical to the standard Moran process, but less trivial digraphs can be used to model hierarchically organized cells, where some mutations in some cells are more likely to be fixed than if others. It is as a matter of fact possible to define graphs where an infinitesimal fitness advantage guarantees fixation, as well as the exact opposite: graphs where a mutation, no matter how advantageous, has zero chance of fixation [98]. An appropriate graph could be used to simulate CSCs, by creating a structure where mutations in some cells are more likely to be fixed than normal.

Birth-death processes are Markov-chains defined by some state variable (often population size in this context) and are limited to taking either a birth step, increasing the state variable, or a death step, decreasing the state variable. This is frequently extended to having multiple types of cells, and thus many different types of birth/death events, with mutation steps connecting the different cell types (Figure 2.5B). An important result from derived using a birth-death process is that high turnover cancers are more vulnerable to mutations [62]. The fixation probability for a single mutant cell can sometimes be solved exactly in this type of model as well, although since it is a more loosely defined category no single expression can be given. The fitness of cell types in a birth death process influences the expected number of that type over time, but does not greatly influence the probability of any particular cell type being discovered [19]. This has implications for the detection of mutants upon diagnosis in CML, where certain mutations may be more likely to be detected without necessarily being more likely to be present (and subsequently cause resistance) [19]. A birth-death

process was also used to show how drug combinations of current drugs can improve outcomes in CML [99]. Yet another example involves a similar stochastic model (as an underlying hidden process in an otherwise deterministic system) was used to examine CSC quiescence in CML, offering one explanation for the common rapid relapse upon ceasing treatment [100].

Branching processes consider individual cells and their lineages. Cells live for some random length of time, and either during their lifetime or upon their death, they produce some deterministic or stochastic number of offspring (Figure 2.5C). Their offspring follow the same rules in turn [101]. Unlike the previous two models, a branching process contains a (phylogenetic) inheritance tree. It takes no major leaps in thinking to see how this connects to how cells typically behave. A branching process can also be modified to create limited growth up to some carrying capacity [86]. Furthermore, much like in the previous model types, it is possible to have one or more types of cells. Using a single type may be enough to model the population growth, whereas a multitype process may aptly represent mutation dynamics [102]. Both an enumerable number of types, for instance a vulnerable and a resistant type, or an infinite number of types are possible [103], which could be used to model some continuous inherited parameter. Types could also be arranged in a hierarchy, where the odds of transitioning from one to the next are structured on a graph [104]. That is commonly featured as an abstract model of compound mutations. The odds of preexisting mutations have a closed solution in simple branching process models [105]. Branching processes have also been used to optimize treatment combinations showing that if there are overlapping resistance mutations treatments will not offer long-term success [104].

2.8.2.2 Spatial models

Somewhat out of scope for this thesis, but nevertheless worth mentioning are models that include the spatial structure of a tumour. That is not to say that leukaemias have no spatial component, merely that they grow already in a matrix designed to support a great deal of proliferative activity and do not form compact solid structures to the same extent.

As they are already carefully studied in computer science and mathematics, cellular automata present an attractive option. In a cellular automaton, cells exist on some grid (frequently two dimensional, but higher or lower dimensions are possible), or more generally on some spatially embedded graph (Figure 2.5D). These models can become very involved and realistic, with the main limitation being the the lack of flexibility in the static grid or graph. This is mitigated somewhat by the tendency of cells to stack reasonably orderly, but unmitigated again by the tendency of cancer cells to break that conformity. A common addition is to model the microenvironment using a continuum model and the cells with a cellular automaton [87].

Finally, there are fully biophysics/biochemistry based simulations [87], which do away with as many approximations as they can to get something qualitatively and quantitatively as similar as possible to real disease. As a category it defies further classification. Obviously, these approaches require a substantial knowledge base to construct, and could easily suffer from parametrization difficulties. The more involved they become the further the computation burden grows as well, which either limits use, or puts further demands on design expertise to apply supercomputer resources parsimoniously. Nevertheless, these models have produced very interesting results in many cancers, especially regarding phenomena such as invasion and interactions with the microenvironment; like nutrient and drug transport [106].

2.8.3 Continuum models

Agent based models can easily become complicated, but that is not to say all stochastic processes defy analysis, which certainly is not true. Exact solutions for many properties, like fixation times, expected population sizes, etc. are known for many standard models, as previously discussed. Another way of analysing a stochastic model is to utilize a continuum approximation. A continuum approximation takes discrete cells counts and discontinuous division and death events, and treats them using differential equations where population sizes are real numbers that change continuously over time. Applications of continuum models are frequent.

For instance, the properties of a cyclic treatment (drug rotation) are in some cases well described by deterministic equations [107]. By clever model design, the same core idea is analyzed from both a stochastic and deterministic viewpoint, providing insight into success-probabilities and population size dynamics of different cancer cell subpopulations respectively. This kind of approach is often viable for branching processes, though some care is needed as there are situations where it might not be accurate [94].

A recurring idea using continuum mechanics is that of a multiple compartment structure, where each compartment holds cells that divide producing either more of themselves or cells in the next downstream compartment. This is how the normal HSC replication manages to output an immense number of cells every day despite HSCs only rarely dividing [16]. A one-way hierarchy of haematopoiesis, whether normal or in a neoplasm, also acts as an evolutionary suppressor, lowering the positive selection from a fitness advantage [18, 98].

2.8.4 Modelling therapy and drug response

Pharmacokinetics, the theory of how drugs are absorbed, distributed and eliminated from the body, is often included as an element of a cancer treatment model. Such an approach has been used more than once to optimize epidermal growth

factor receptor (EGFR) –inhibitor therapy in lung cancers using a stochastic birth-death process with the influence of drugs determined using pharmacokinetics [108, 109]. It would appear that EGFR mutated lung cancer may respond well to intermittent high dose therapy with continuous low dose therapy.

2.8.5 Practical notes

When resorting to simulations, most models are subject to varying degrees of approximation: tradeoffs are made between accuracy, speed, thoroughness, and predictive utility. Some specific cases are discussed in the Methods chapter. Commonly, very rare events are treated as impossible; the infinite sites assumption is one such example. While often applicable, some care needs to be taken to distinguish between rare events of interest, and rare events that are legitimately negligible. E.g. including mutations, but neglecting the possibility of two SNVs of interest happening in one cell division may be fairly clear cut. The latter may be over 1 000 000 times less likely, and mutations are rare to start with. All cases are not so clear cut however, and there is obvious way to draw the line.

In any continuum model, differential equations will almost always be solved numerically. Doing so is an entire field of study, and the intricacies will not be discussed further here. Nevertheless, the same tradeoffs occur here as well.

Table 2.1: CML Disease phases

Phase	Symptoms	Prognosis	Treatment
Chronic phase	Typically mild or absent • Fatigue • Anaemia • Splenomegaly • Weight loss	Indefinite progression free state often possible. Transition to BC in 2 to 5 years without treatment.	TKIs, most commonly starting with imatinib. allo-HSCT if there is a good match.
Accelerated phase	• Fatigue • Worsening anaemia • Splenomegaly • Weight loss	Median survival on the order of 8 years, but highly variable.	Alternate TKIs or dose escalation. allo-HSCT
Blastic phase (Blast crisis)	Typically severe • Thrombosis • Severe anaemia • Secondary infections	Median survival less than 2 years.	Alternate TKIs or dose escalation. allo-HSCT if possible. Chemotherapy or radiation.

Note: Data compiled from [21, 28, 29].

Table 2.2: CML drugs

TKI	Status	Indications	Contraindications	Adverse effects
Imatinib	1st gen. TKI	• First-line treatment • Failure of IFN–α	• Any one of several kinase domain (KD) SNVs.	• Weight gain and edema • Fatigue • Aches
Nilotinib	2nd gen. TKI	• First-line treatment • Treatment failure • Young patient	• Mutations: Y253H, E255K/V, T315I, or F359C/V • Diabetes • Cardiovascular issues	• Hyperglycemia • Vaso-occlusive events
Dasatinib	2nd gen. TKI	• First-line treatment • Treatment failure • Young patient	• Mutations: V299L, T315I, or F317L • Patient on anticoagulant • History of pleural effusions	• Pulmonary arterial hypertension
Bosutinib	2nd gen. TKI	• First-line treatment • Treatment failure • Young patient	• Mutations: V299L, T315I, or F317L • Renal dysfunction • Gastric issues	• Gastro–intestinal • Hepatic • Renal
Ponatinib	3rd gen. TKI	• T315I presence	• Cardiovascular issues • Preexisting cardiopulmonary disease	• Thrombotic events • Pancreatitis
Asciminib	Experimental allosteric inhibitor	• T315I detected • Under evaluation as second–line option	• High lipase?	• Liver issues • Pancreas issues
Axitinib	Experimental TKI (Used in RCC)	• Large fraction of T315I–positive cells	• Cardiovascular issues	• Thrombotic events

Note: There are innumerable experimental drugs, as well as drugs approved only in some regions. The listed few are present because they are discussed further in this work. However, rebastinib, discussed in Paper I, is excluded from this list as research on it has been discontinued. Data adapted from [29, 50].

Chapter 3

Aims and objectives

The development of new drugs is only one possible path towards providing new treatment options for CML patients. Using the drugs we have in the most effective manner possible is another, perhaps somewhat overlooked, aspect. This thesis aims to investigate whether currently available drugs, and/or promising candidate drugs, could be used to form treatment regiments that are superior in some respect to those currently used. Of particular concern is:

1. Drug rotations, where two or more drugs are used in an interleaved manner. This is the most likely alternate treatment strategy to be adopted in practice; there are even some case studies and trials [110–112]. Drug rotations are favoured since changing from one drug to another that works is a somewhat risky but not outrageously dangerous treatment decision.

2. Drug combinations, especially involving standard TKIs and upcoming allosteric inhibitors like GNF-2/5 and asciminib (ABL001). Such combinations likely have a greater potential than combinations of regular ATP binding site TKIs since the drugs do not compete for binding to the same extent.

3. Drug holidays, where treatment is suspended for a while only to be resumed later. Such an approach may resensitise the tumour to the drug. It may also provide some relief if side effects are bad. Finally, patients may be forced to stop treatment for various reasons, hence knowing the consequences, good or bad, of such a move is desirable.

To study this, we employed a combination of in silico models of evolution, with complementary experiments on CML cell lines.

Computer modelling

A major part was to construct tools for in silico testing of drug treatment protocols. The original goal was to use as much information about the sensitivity of known BCR-ABL1 resistance mutations as possible to provide a realistic model of the response. The models were simplified somewhat in later on however, to capture the most vital effects without extraneous clutter. A secondary aim was to create the tools needed to analyse certain cell line experiments.

Cell line experiments

If our modelling were to uncover something promising, the next step would be to run some initial test on TKI sensitive CML cell lines. The main things tested were verifying our assumptions about drug interactions for Paper III, and testing the effects of drug rotations, predicted in Paper I, and tested in Paper II after we managed to develop an experimental set-up for it.

Chapter 4

Methods

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4.1 Cell line experiments

Two Ph+ cell lines were chosen for experiments, K562 and KCL-22. K562 was the first CML cell line ever to be isolated [113]. It has been reported to become resistant to TKIs through long term gradual dose escalation [68,114,115], but not very easily, and is quite sensitive to drugs. KCL-22, like K562 was also isolated from a woman in BC [116]. It has two copies of the Ph–chromosome, and is relatively genetically unstable, likely owing to a loss of function in human mutL homologue 1 (hMLH1), a DNA mismatch repair protein [117]. Experimental investigations have also indicated that KCL-22 cells acquire resistance rather quickly, and a T315I positive cells can purportedly be produced from a single, high concentration TKI exposure [118,119].

Another cell line worth mentioning is K812. It is known for being extremely sensitive to BCR-ABL1 TKIs, and very challenging to induce any kind of resistance into [68]. Thus, we did not choose it for our experiments. In addition, it happened that K562 was already sensitive enough to drugs to fill the role of a highly sensitive cell line (Paper II).

4.1.1 Assays

The two assays we used most frequently were various forms of cell counting with a trypan blue stain, and viability measurements with a tetrazole dye (MTS). The former falls in a category of vital stains. Trypan blue is an ionic dye that cannot penetrate the cell membrane. A broken cell membrane allows the dye to enter, colouring the cell blue, and is also a certain sign of a dead cell [120]. Hence, it can be used visually to distinguish dead cells from living under a microscope. It is nevertheless also possible for a cell to be dying, but not yet permeable to trypan blue. Thus relying on only a vital stain is less sensitive for detecting drug toxicity effects than other assays that also identify apoptosis markers (such as propidium iodide/Annexin V [121]). Be that as it may, by collecting timeline data where sequential results carry information about the true health-status of the culture, this drawback could be summarily circumvented. MTS, on the other hand, is a compound that is enzymatically reduced to a purple formazan dye in living cells. Since the reaction is NADH dependent, it only happens in cells with an active metabolism. The amount of colour produced in a certain time frame is thus a measurement of the metabolic activity, and by extension the number of living cells [120]. The drawback of this technique is its more destructive nature, as cells cultured with the dye are not suited for continued experiments[1]. It is also difficult to get absolute reproducibility between experiments, as the colour depends on incubation time. Therefore, careful controls, or an experimental design that only requires relative measurements, is required.

There were four principal experiments done throughout. Not all of them were included in the papers, and not every variation is elaborated on here for the sake of brevity. Instead, I will focus on the main components required for the drug synergy assay for ATP–site and allosteric drugs, as described in Paper III and the drug rotation experiment, as described in Paper II.

4.1.2 Drug efficacy

As some of experiments relied upon using a TKI dose where CML cell lines would still grow, but do so slowly, it was necessary to first determine a dose-response relationship (Equation 2.4). To this effect, CML cell lines were cultured in a 96

[1] While the same is true for trypan blue, it requires only small (10 µL) samples, thus is not very disruptive for small scale experiments. MTS assays by contrast requires typically at least an order of magnitude more cell culture.

well plate in the presence of a range of concentrations of an inhibitor. Naturally, the amount of growth in each well will depend on the TKI concentration and its efficacy. After 2 to 3 days, the number of viable cells in each well was measured by adding MTS. It is converted to a coloured formazan in proportion to the number of living cells, thus measuring the absorbance provides a measurement of the living population. The absorbance and TKI concentrations could then be used to find the IC_{50} and hill-coefficient by fitting to a logistic curve (e.g. Figure 2.4). If drug concentrations were appropriately chosen, the plate will range from almost no growth to practically uninhibited growth. A twofold dilution series across 12 wells results in a ratio of 1:2048 between the highest and lowest concentration, providing almost two orders of magnitude headroom in each direction from the centre. Even so, the experiments occasionally had to be redone to get the range correctly, with the IC_{50} near the centre of the concentration range, as the available results in Ba/F3 did not generalize to other cell lines. Capturing the range of effects from almost none to total inhibition provides the most accurate measurement.

Example procedure 10^4 KCL-22 cells per well were seeded in 100 µL medium (RPMI 1640, 10 % fetal bovine serum (FBS), 1 % PenStrep) into a 96 well plate prepared in advance with imatinib ranging from $4\,\mathrm{nmol\,dm^{-3}}$ to $8192\,\mathrm{nmol\,dm^{-3}}$, each in triplicate. The plate was incubated at 37 °C, 5 % CO_2 for 2 days, after which 20 µL MTS (Celltiter 96) was added to each well and left to incubate for 3 h. Finally, the absorbance at 490 nm was measured on a plate reader.

4.1.3 Drug synergy

Some drug synergy trials were carried out using the Chou-Talalay combination index (CI) method [122,123]. It is similar to the efficacy assay described above, but in addition to individually testing each drug it also involves an assay using some fixed concentration-ratio combination of two drugs. This is enough to identify synergistic and or antagonistic effects, with a comparatively simple experiment. In the case of mutually exclusive drugs, i.e. drugs where only one could bind to the target at any one time, Equation 2.4 becomes

$$f_v = \frac{1}{1 + \left(\frac{[I]^{(\mathrm{A})}}{\mathrm{IC}_{50}{}^{(\mathrm{A})}} + \frac{[I]^{(\mathrm{B})}}{\mathrm{IC}_{50}{}^{(\mathrm{B})}} \right)^m}. \tag{4.1}$$

The CI for a drug combination is the amount of inhibitors required to achieve some effect in a drug combination, relative to the inhibitor concentrations required to reach that same effect on their own. Assume we have a drug combination of 1

part drug A to 3 parts drug B. If the total concentration required to reach half maximum inhibition is $IC_{50}{}^{(1,2)}$, then the concentration of the constituents are

$$C_A = \frac{1}{4} IC_{50}{}^{(A,B)} \tag{4.2}$$

$$C_B = \frac{3}{4} IC_{50}{}^{(A,B)}. \tag{4.3}$$

From this, we can calculate the CI at 50 % inhibition as

$$CI_{50} = \frac{C_A}{IC_{50}{}^{(A)}} + \frac{C_B}{IC_{50}{}^{(B)}}. \tag{4.4}$$

With the same methodology the CI can be calculated for any effect and it is possible that a drug combination may be synergistic at low concentrations and antagonistic at higher concentrations, or vice versa. A CI value below 1 indicates synergy, whereas a value above 1 indicates antagonism. What we need is the ability to calculate the drug concentration required to achieve a certain effect (i.e. the parameters of Equation 2.4) for both inhibitors on their own, as well as for the combination. This is why a Chou-Talalay CI method trial takes the form of a series of the previously described drug efficacy experiments.

Example procedure Drug efficacy assays, as previously described, were performed for dasatinib ($0.001\,95\,\mathrm{nmol\,dm^{-3}}$ to $4\,\mathrm{nmol\,dm^{-3}}$), and asciminib ($0.006\,25\,\mathrm{nmol\,dm^{-3}}$ to $12.8\,\mathrm{nmol\,dm^{-3}}$), followed by a drug efficacy assay using a dasatinib–asciminib concentration ratio of 65:262 ($0.0159\,\mathrm{nmol\,dm^{-3}}$ to $32.7\,\mathrm{nmol\,dm^{-3}}$). IC_{50} values and hill coefficients were estimated with a four parameter logistic curve using Stan [124], and the combination index was calculated [123].

4.1.4 Resistance generation

As part of responding to reviews for Paper III, we attempted to generate drug resistance KCL-22 cells using the procedure from [118, 119], so that we could measure drug synergy effects in resistant cells as well. The authors report that KCL-22 cells, exposed to a lethal (but not extreme) dose of any first or second generation TKI initially die off but, after two weeks or so, regrows as a T315I mutated variant. The method is purportedly very selective towards generating T315I mutants, possibly as a consequence of the high mutation rate of KCL-22 and the very high degree of resistance provided by T315I. Thus, the cells, while dying, would produce a number of resistant variants and the most resistant, i.e. T315I, would outcompete the rest leading to the selectivity. We attempted this procedure while responding to reviews for Paper III, albeit unsuccessfully: No resistant cells were produced, and the KCL-22 cells did not survive imatinib exposure. Normally, the cells would have regrown appreciably after 20 days. The experiment was not repeated due to time constraints.

Example procedure Two 24 well plates were seeded with 10^6 KCL-22 cells in 1 mL medium and 2, 5, 8 and 12 µmol dm^{-3} imatinib, each in quadruplicate. Population sizes were measured every other day using a LUNA-II cell counter with a 1:1 trypan blue stain (0.4 %). After 12 or 13 days, 0.5 mL medium was added to restore the total volume, as some medium was lost to evaporation. After 33 or 35 days, the cultures with most living cells were diluted to 10 mL in medium supplemented with 1 µmol dm^{-3} imatinib, as they were not recovering in number as expected, but the cultures never regrew.

4.1.5 Drug rotation trial

We predicted in Paper I that drug rotations, especially those involving ponatinib, should reduce the risk of acquiring resistance mutations. This means that, on average, cells should become resistant somewhat slower than they would to a monodrug protocol and he drug rotation experiment was designed to examine such effects. Of course, in reality mutations are not the only possible adaptation, merely the most common clinically. The idea was to track the growth rate of the cells and see when and how quickly it increased as the cells started adapting to the drugs. To enable such tracking, the cells would have to be cultured at a drug concentration where they could grow, albeit slowly. This is also conceptually consistent with how CSCs may be affected, but not killed off, by TKIs. The method described in Paper V was designed specifically for analyzing this experiment, as it exhibits the regular counting and consistent growth that the algorithm works best with.

In Paper II we performed this using imatinib–ponatinib rotations in both KCL-22 and K562 cells. Switching drug required some form of washing procedure to get rid of the old drug, which combined nicely with reseeding down to a lower concentration to remain in the exponential growth phase (so that evolution would not be limited by carrying capacity in some unexpected way). See Figure 4.1 for a schematic illustration of the procedure.

Example procedure 10^5 KCL-22 cells were seeded in 1 mL medium in two 24 well plate, in the presence of either imatinib (240 nmol dm^{-3}) or ponatinib (0.37 nmol dm^{-3}), each in quadruplicate. Each well was counted every other day using an automated cell counter (LUNA-II) and a 1:1 trypan blue (0.4 %) stain. After 6 days, the contents of each well was transferred to an eppendorf tube and centrifuged at 300 g. The supernatant was discarded and the pellet resuspended in medium, before being spun down again and resuspended in inhibitor-doped medium, switching the inhibitor in half of the imatinib and ponatinib wells (Figure 4.2). At this points, the cells were counted, and subsequently diluted to 10^5 cells in 1 mL medium once more in a fresh 24 well plate. This procedure was repeated 6 times, for a total of 36 days and three cycles in the drug rotation

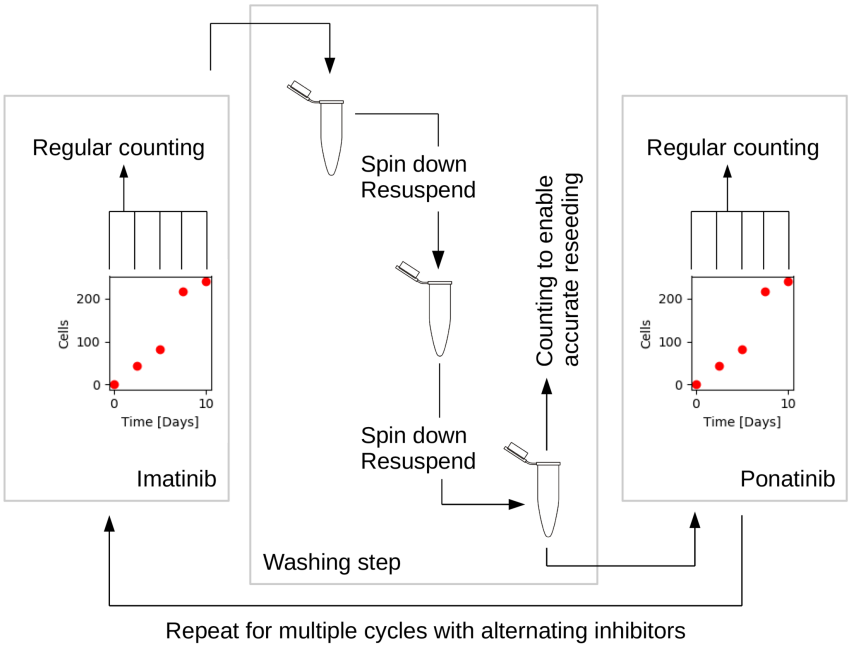

Figure 4.1: Imatinib–ponatinib (IM–PO) drug experimental design.

wells. Upon each washing cycle, all remaining cells were frozen for possible later analysis.

4.2 Modelling approaches

A significant portion of this thesis involved simulations of stochastic processes. Simulations were used as they greatly simplify getting quantitative results from complex models. Unlike an analytical solution, which often cannot be arrived at without designing a model such that it is possible, simulations simply require a model and some computing power. Fortunately:

- powerful computers are more abundant now than ever and
- sampling a single model many times is easily parallelizable, which gets around the main difficulty in using powerful computers.

The more random the process, the more samples are required to accurately represent the outcomes. Furthermore, stochasticity can create effects that are not accurately represented in continuum approximations; meaning simulations might be the only viable approach [94].

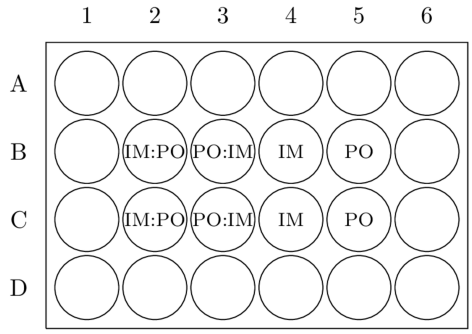

Figure 4.2: Imatinib–ponatinib (IM–PO) drug rotation plate setup. The wells labelled IM:PO or PO:IM switch from one inhibitor to the other with each washing–reseeding cycle; the difference between them being the starting inhibitor. The wells labelled IM or PO are washed and reseeded in the same way, but maintain the same inhibitor throughout the experiment. All of the surrounding empty wells were filled with medium to attenuate evaporation.

4.2.1 Normalization of treatment effects

A recurring theme in any of the papers in this thesis that deals with experimentally derived properties of drugs is how to normalize treatment effects. Currently available TKIs range in affinity by almost three orders of magnitude, with imatinib IC_{50}s in the $\mu mol\,dm^{-3}$ range and dasatinib IC_{50}s in the $nmol\,dm^{-3}$ range. There is a great deal of variation in blood plasma concentrations, and those measurements do not correlate well to neither observed CML anti-proliferation or to BCR-ABL1 IC_{50}s. At a surface level though, when comparing plasma concentrations with BCR-ABL1 affinities it appears that some drugs are vastly more effective than others. In Paper III we argue

> "... normalise the doses of all drugs so that they are equally effective [...] by assuming that standard drug doses have been calibrated to minimise side effects while inhibiting proliferation enough to be an effective treatment."

which is one way of getting around this issue. The correctness of this approach is genuinely quite hard to determine, and so we also perform all analysis using unadjusted values whenever possible. In Paper I we instead simply perform simulations for a range of different drug doses (or technically degrees of inhibition caused by drugs).

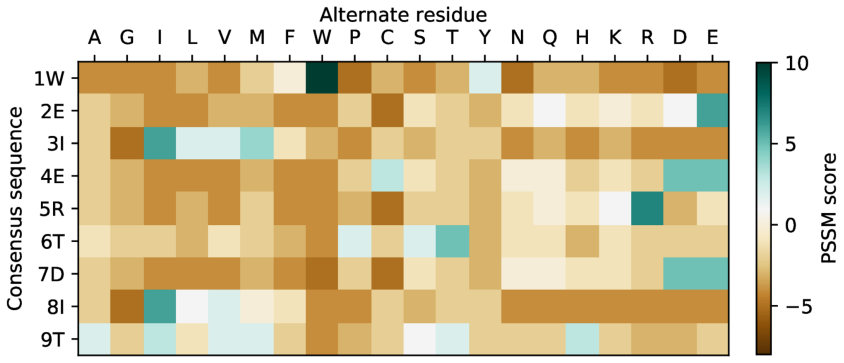

Figure 4.3: Position specific scoring matrix for the first 9 positions in the catalytic domain of ABL1 (cd05052), derived from sequence homology [125]. Note how most residues are quite conserved, as indicated by having only one high scoring option.

4.2.2 Analytical and continuum approaches

In Paper I we derive a formula for how the timing ratio between two drugs in a drug rotation influences the expected time until a resistance mutation emerges, by treating both the cell population and the drug rotation as a continuum. It provides a shortcut means of optimizing the timing ratio, as performing simulations for the same information is much more arduous, and clarifies how the relative fitness of mutations influences the outcome as the fitness varies with the timing ratio.

A continuum approximation was also initially used to derive individual growth rates for each mutant in Paper I. By asserting that the frequency with which each amino acid appears in a certain position in homologous proteins across all domains of life is representative of the efficiency of action in the resulting protein, we found a way of calculating the growth rates of mutants from a position specific scoring matrix (PSSM) (Figure 4.3) using a continuum approximation. Ultimately, this method was not used further on. The derived rates were very similar, a consequence of the approximation, and the fundamental assumption that variations in homology should match the fitness effects on the enzyme is not entirely accurate.

In Paper IV assuming a large population size enables treating a set of parameters as a probability distribution, i.e.

$$\{x_0, x_1, ...x_N\} \to f_X(x) \text{ as } N \to \infty. \tag{4.5}$$

The probability distribution is then evolved using a partial differential equation (PDE).

$$\frac{\partial f_{X,t}(x)}{\partial t} = (f_{X,t}\lambda * f_Y)(x) - f_{X,t}(x)\int \lambda(\xi) f_{X,t}(\xi) d\xi \tag{4.6}$$

Note that "$*$" is the convolution operator. This approach enabled vastly quicker simulation for parts of the parametrization and was critical to make the problem tractable.

In Paper III we forego detailed models of evolution and instead focus on wrangling Equation 4.1 in presence of drug doses whose concentrations follow steady–state pharmacokinetics. A BCR-ABL1 mutation is only a threat so successful treatment if it lowers the effects of TKIs such that the mutated cells grow faster than before. So, for simplification, we exploit that to judge whether a drug combination can effectively suppress a mutation. This mostly depends by whether the mutant grows faster under given treatment conditions; which can be evaluated using Equation 4.1. Using that approach, every reasonable way of taking combinations of standard TKIs and axitinib or asciminib was tested. After identifying the best dosing ratio $x \in [0,1]$ for treating mutation free cancer cells, it is possible to extend to combine available mutation–IC_{50} correlation tables to produce the same data for a drug combination by solving

$$\frac{1}{2} = \frac{1}{1 + \left(\frac{Cx}{{IC_{50}}^{(A)}} + \frac{C(1-x)}{{IC_{50}}^{(B)}}\right)^m} \tag{4.7}$$

for the combined IC_{50}: C. That needs to be repeated for every different mutation, as ${IC_{50}}^{(A)}$ and ${IC_{50}}^{(B)}$ changes, but in effect makes it possible to produce information similar to what is shown in Figure 2.2 for an arbitrary drug combination.

4.2.3 Implementation of the stochastic model in Paper I

Consider a population of two types of cell types A and B, with N_A and N_B of each. Each cell type reproduces at a rate r_A and r_B, and typically produces more of their own kind but with some tiny probability μ they may produce offspring of the other type. If we take a small timestep (small compared to r_A and r_B), we should expect a binomially distributed, $\mathcal{B}(N,p)$, number of new cells:

$$N_{A,t+dt} = N_{A,t} + \mathcal{B}(N_{A,t}, r_A). \tag{4.8}$$

In the same way, a small fraction of those new cells mutate (also binomial but with probability μ), instead becoming type B. Naturally, the same happens with the types inverted. This approach is very straightforward to simulate. However, it does not accurately model the (rare) possibility of multiple divisions in one timestep, which is implied to exist by the model of cells dividing at

some rate. This approach is similar to an algorithm called tau–leaping, however tau–leaping typically implements an adaptive timestep to minimize inaccuracy, and the transition to exact solutions under certain circumstances. The practical reason for adopting this model design is that it is very computationally efficient. In each timestep taken, we only need to evaluate a binomial random number for each extant cell-type. The types in Paper I were defined by their genotype. Because mutations are rare, only a handful of types would typically be present in simulation at any moment. Thus, the time the computation takes scales almost linearly with the number of timesteps. Furthermore, longer timesteps can be used to get even more speed at the cost of accuracy.

Paper II, Paper IV and Paper V use Gillespie's algorithm (or equivalent) for stochastic simulations. It is explained in further detail below.

4.3 The Gillespie algorithm

Gillespie's algorithm is a method of exactly simulating certain stochastic systems. It was popularized by its use in simulating the master equations of stochastic kinetics [126]. As that is a tangible subject, I will resort to a chemistry example in for its introduction below, followed by a transition into how it is used to simulate evolutionary models. Consider a simple reversible dimerisation equilibrium

$A_2 \rightleftharpoons 2A$

We can produce the differential equations describing how each chemical species (i.e. A and A_2) changes over time

$$\frac{d[A_2]}{dt} = -k_d[A_2] + k_f[A]^2 \tag{4.9}$$

$$\frac{d[A]}{dt} = 2k_d[A_2] - 2k_f[A]^2 \tag{4.10}$$

and this set of equations is called the chemical master equation. k_f and k_d are the rate constants for formation and decomposition respectively.

If we have a large amount of reactants (A, A_2) the differential equations will provide a very good approximation. However, when the concentrations are very low, the continuum approximation is quite inappropriate, as in reality, we always have an integer number of molecules and individual decomposition reactions are events with a specific locality in time. For a more realistic representation, we need to integrate it including the stochastic dynamics.

As individual decomposition reactions are not in any way coupled, it follows that the time between two reactions in the system is exponentially distributed. The same is true for the aggregation reaction, so long as the system is well mixed. Therefore, we can simulate it by first selecting at random a waiting time based on the odds that either event will happen. We then select one the events, either a dimer formation, or a decomposition, based on the rate constants and the number

Listing 4.1: Example Nim implementation of Gillespie's algorithm for the system in Equation 4.9 and 4.10

```
import math, random, strformat

let
  t_end = 10.0
  k_f   = 4.0e3
  k_d   = 1.0e3
var
  t    = 0.0
  n_A  = 100
  n_A2 = 0
randomize()

echo "time\tn_A\tn_A2" # print results like a .tsv file
echo &"{t:3.3f}\t{n_A}\t{n_A2}"
while t < t_end:
  let total_rate = n_A.float/k_d + n_A2.float*n_A2.float/k_f
  t += -total_rate*ln(rand(1.0))
  if rand(1.0) < n_A.float/k_d/total_rate:
    n_A -= 2
    n_A2 += 1
  else:
    n_A += 2
    n_A2 -= 1
  echo &"{t:3.3f}\t{n_A}\t{n_A2}"
```

of possible reactions of each type. This process is then repeated until some end criterion is reached, for instance a stopping time (Listing 4.1).

4.3.1 Application to evolution

A culture of cells can also be described by a system of master equations. Principally, there are two types of events: Cell division, producing 1 new cell (or alternatively killing one and producing two), and cell death, removing one cell. For completeness, let's also include a rare odds of producing a mutant cell (producing two mutants at once is neglected due to its nigh infinitesimal probability). For two types of cells named A and B, with the infinite sites assumption disallowing backward mutations from B to A, a culture of cells could be represented by

$$\mathrm{A} \longrightarrow 2\,\mathrm{A}$$
$$\mathrm{A} \longrightarrow \mathrm{A} + \mathrm{B}$$
$$\mathrm{B} \longrightarrow 2\,\mathrm{B}$$
$$\mathrm{A} \longrightarrow$$
$$\mathrm{B} \longrightarrow$$

Note how, unlike in chemistry, it is entirely feasible for this system to have "reactions" that produce no products. For growth rates k_A, k_B and a shared death rate k_d, we get

$$\frac{d[\mathrm{A}]}{dt} = k_A[\mathrm{A}] - \mu k_A[\mathrm{A}] - k_d[\mathrm{A}] \tag{4.11}$$

$$\frac{d[\mathrm{B}]}{dt} = k_B[\mathrm{B}] + \mu k_A[\mathrm{A}] - k_d[\mathrm{B}] \tag{4.12}$$

which can be simulated in much the same ways as described in listing 4.1, so I will refrain from detailing it further.

4.3.2 Infinite types

In Paper IV, the master equation consists of an infinite system of equations: one for each value of a continuous expression. While it is not possible to write down such an equation in a closed form, it can nonetheless be simulated in a straightforward manner (Listing 4.2). Gillespie's algorithm requires knowing the rate with which each individual reaction can happen. By realizing that only cells that exist can undergo events of any type, we only actually need to consider the equation describing those particular cells at any one time. In practice, this is realized by keeping a list of the birth and death rates for each cell. Its sum is the rate of all events, and it can be used to select an event proportional to the rates.

4.3.3 Other technical aspects

An alternative simulation method, the next-reaction-method, was used behind the scenes in Paper V (and Paper II by extension), which reduces the computational burden by cleverly reusing random variables [127]. It's functionally equivalent to Gillespie, but often faster.

There are exact solutions for extending the Gillespie algorithm to time dependent rates, but I opted for a simpler numerical approach to approximate it. The rate in the future can be estimated in the same way midpoint method forward integration is done, which turns out to be a good approximation if events are frequent compared to the rate of change in the rate constants (Figure 4.4). In a model with only growth, a naive implementation

```
proc rate(t: float): float =
  # return the growth rate at time t
  ...

var N = ... # starting population

while t < t_end:
  growthRate = rate(t)
  t += -totalRate*log(rand()) # sampling the exponential distribution
  inc N
```

Listing 4.2: Unoptimized Nim pseudo-implementation of infinite master equation Gillespie simulating adaptation via change in the expression parameter (Paper IV).

```
proc growthRate(x: seq[float]): seq[float] =
  # function returning growth rate from expression parameter
  # takes a sequence of parameters and applies function to each of them
  ...

proc init(cells: var seq[float]) =
  # fill the list of cells
  # each value represents one cell's expression parameter
  ...

proc randomNoise(): float =
  # return a random change in expression upon cell division
  ...

let
  endTime          = 10
  carryingCapacity = 2e6
var
  cells: seq[float]
  t = 0

cells.init()
while t < t_end:
  let
    growthRates = cells.growthRate()
    totalRate = growthRates.sum + cells.len*(cells.len - 1)/carryingCapacity
  t += -totalRate*log(rand())
  if rand() < n_A/k_d/total_rate:
    # select a parent cell with odds proportional to growth rate
    let parent = sample(growthRates, growthRates.cumsummed)
    cells.add(parent + randomNoise())
  else:
    # select a dying cell randomly
    let dying = rand(cells.len - 1)
    cells.del(dying)
```

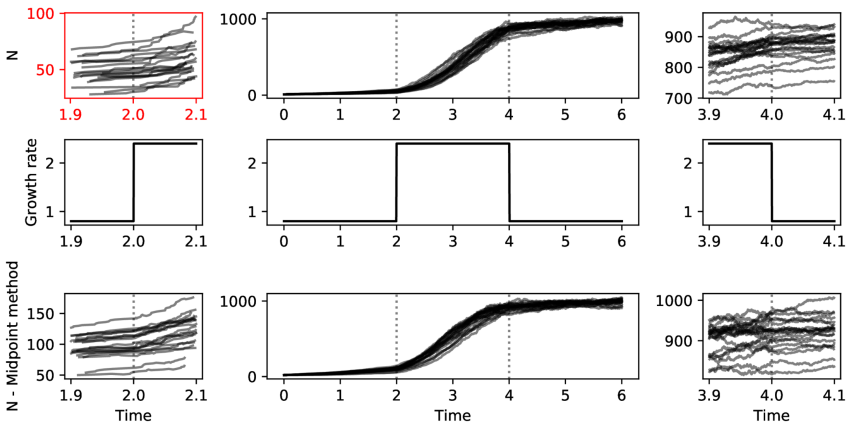

Figure 4.4: Stochastic simulations of logistic growth with a variable growth rate. Using a rate from the future in Gillespie's algorithm in a manner similar to the midpoint method forward integration improves its response to a rapidly changing rate when the total event rate is low. Compare the significant delay after the growth rate changes at Time 2 in the top left panel (red) against the bottom left panel, where no observable delay is present.

where the growth rate is sampled at the current time, note how if the growth rate changes during the waiting time for the next event that will not be accurately simulated. However, since the average waiting time between events is the inverse total rate we can estimate the waiting time and use a rate from the future:

```
while t < t_end:
  growthRate = rate(t + 1/rate(t)/2)
  ...
```

which removes the bias induced by waiting past when the rate changes. The code is somewhat more involved if deaths are included, but is still very tractable. Also, in Figure 4.4 notice that there is only a significant difference at time 2 (leftmost column), where the population sizes, and thus the rate of growth events is low. At time 4 (rightmost column), there is no discernible difference as shorter waiting times shrink the size of the error.

Finally, a quick note on scaling. Since the number of events (births, deaths, ...) in some time segment is proportional to the number of cells (in the simple case), simulating for a time t is proportional to the number of cells, and the time ($O(Nt)$). With the infinite master equation system in Paper IV, this is worsened by needing to, in practice, treat every cell individually. While the code in listing 4.2 can be optimized by keeping a running total of the growth rates, selecting a random parent cell for birth events is $O(N)$. Thus, its scaling is $O(N^2t)$, rendering it difficult to simulate a large population of cells.

Table 4.1: Likelihood when observing **BRB**

Balls	Ways to produce		Likelihood
RRR	0	We have seen **B** so this is impossible	0
RRB	2	When picking **B**, there is only one option. When picking **R** it could happen it two ways.	$1/3$
RBB	4	When picking **R**, there is only one option. When picking **B** it could happen it two ways and we do that twice	$2/3$
BBB	0	We have seen **R** so this is impossible	0

Note: Assuming a uniform prior, i.e. black and red balls are equally likely from the start.

4.4 Approximate Bayesian computation

Approximate Bayesian computation (ABC) are a class of numerical techniques for likelihood-free inference; i.e. answering the question

> *"Given some measurements, and a model of how they are produced, what model parameters could have produced those measurements?"*

in cases where it cannot be done analytically [128]. The likelihood of a set of parameters is the relative number of ways that they could have produced the given data. Consider the simple and ever popular example of an opaque bag of different coloured balls. Say we know it holds three balls, each of them red or black but the number of each colour is unknown. We put our hand in the bag and pull out one ball at random, and we get a black ball **B**. It is then put back into the bag, and a new sample is taken. This is repeated once more, giving the observation series **BRB**. What colours are the set of balls in the bag? That set of colours is our model parameters, i.e. the hidden knowledge that determines observations behind the scenes. The truth is we cannot know, but we can say what is most likely, namely two black and one red (Table 4.1).

I should also introduce the idea of priors. Consider that you knew beforehand that the factory that produces bag-experiment balls makes one black ball for every million red ones, and the experiment is done with a random selection you purchased. Suddenly, the likelihood of the set of balls in the bag being **RBB** is very low, and consequently **RRB** will have the highest likelihood. The likelihoods in this case can of course be treated formally [129], but this nonetheless illustrates the importance of the prior.

The likelihood for an arbitrary problem can only be solved analytically in special cases (which nevertheless are a hugely important part of statistics and

probability theory). However, being able to solve it numerically imparts a great deal of freedom in model design as we no longer have to make sometimes conservative assumptions to arrive at a solution. The beauty of ABC techniques lies their ability to find the likelihoods for *any* model, we need only be capable of simulating observations. In practice, likelihood-free inference often takes the form of inverting models.

In ABC, a forward model, which simulates measurements from some hidden parameters, is used to answer the opposite question: what parameter distribution reproduces some given measurements? This is very advantageous as forward models are typically easier to construct from first principles. Take for instance the model in Paper V. There we start from an assumption that suspension cells normally follow a logistic growth curve, a common approach. The growth rate is then changed to be time-dependent, as it is one possible way to model the cancer cells adapting to the environment. Finally, a way of simulating experimental measurements was added. Starting as we did by assuming core ideas of a model can be far more practical than trying to identify some transformation of experimental data that provides the same information, as prior knowledge of how the system under study is expected to behave can be used.

We used ABC for model parametrization, and for analysis of experiments. More specifically, sequential Monte-Carlo (SMC) (also known as particle filtering) methods were used. This approach models the likelihood as a set of particles, which are evaluated and kept based on how well they fit the observation. After several selection-replication iterations where particles that agree with the measurements are more likely to be kept, the final set of particles represent the parameter distribution (Figure 4.5). There is an appealing symmetry to modelling growth and death of cells with replication and removal of particles, though beyond pleasing design it offers no additional advantages.

The main drawback of ABC techniques is the high computational cost, since in non-continuous systems, only relatively inefficient sampling techniques are available and the model has to be evaluated tens of thousands of times[2]. However, as computational power consistently grows, and because stochastic sampling techniques are very easily parallelizable this issue can be glossed over within reason. So long as gathering the data is significantly more arduous than running the simulations, the point is moot.

Additionally, being able to fit a model to data does not necessarily imply that it accurately describes what is going on. The model has to be independently reasonable and motivated by domain-specific knowledge. Another risk is overfitting, which is when model parameters contain far more information than can be

[2]For non–pathological continuous forward models, very efficient sampling techniques such as Hamiltonian Monte-Carlo are available [130]. These were used in analysing the drug efficacy experiments.

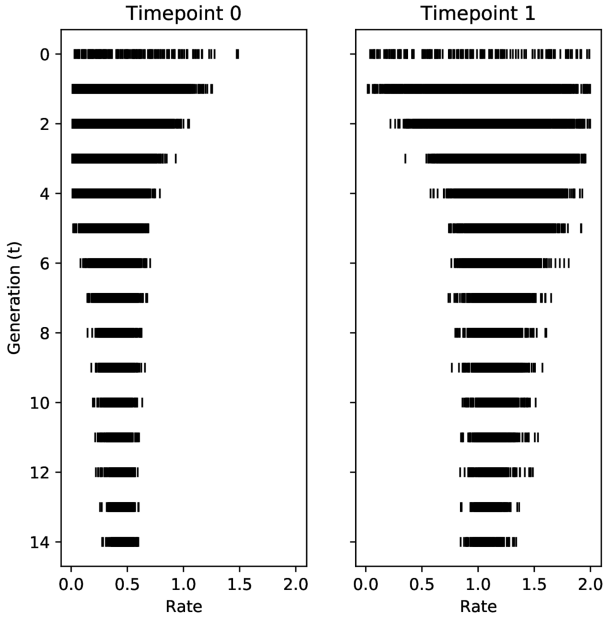

Figure 4.5: Example of sequential monte carlo particles (vertical lines) gradually converging upon the posterior likelihood after several selection–replication iterations (generations). The specific model relates to Paper II, thus Timepoint 0 and Timepoint 1 represent a piecewise linear growth rate.

justified by the data. It can result in random noise being incorporated into the model leading to inaccurate predictions.

In Paper IV, ABC was used to parametrize a model of gradual adaptation mediated by increasing protein expression. Paper V includes it as part of a tool for analyzing experiments; the time dependent growth rate in a suspension cell culture is calculated from a series of cell counts.

Chapter 5

Summary and Discussion

Two published articles and three manuscripts were produced within the scope of the thesis. Here follows a discussion of the main results from each of them. The ordering is non-chronological for the sake of narrative flow. Additionally, some minor notes on otherwise unpublished research are discussed as they add useful information with regards to this thesis.

Paper I

Stochastic modelling of tyrosine kinase inhibitor rotation therapy in chronic myeloid leukaemia [131]

In this project, we designed a constant population size model of CML CSCs growing under the influence of TKIs. The genome subject to resistance mutations is explicitly modelled, and the growth of mutants under treatment was parametrized using available data from Ba/F3 cells [38]. The model involved taking small timesteps producing a binomially distributed number of children for each genotype present in the population, as described in more detail in the Methods chapter. Each reproduction of the modelled genome had some small chance of producing mutants, with silent and missense mutations that result in a drug resistant phenotype being explicitly included.

We then tested two-drug drug rotation protocols on this model, i.e. a treatment protocol which blindly switches back and fourth between two drugs using some fixed periods and dosages. Some extra attention was put towards imatinib–nilotinib as well as a bosutinib–ponatinib rotations, as there were observations from a clinical trial and case study. However, every possible pair was tested. Our results indicate that a drug rotation with well chosen drugs can prolong the average time before mutant cells constitute at least half of the neoplasm by about 10 %. Choosing drugs with distinctly different resistance mutation spectra is imperative for this effect, validating such predictions [107] using data for current TKI. In practice, this means that the biggest effects are to be held from drug rotations involving ponatinib. The timing ratio was also important. The optimum resistance-delaying effect may require using one drug for longer than the other, but in most cases the optimum was not more skewed than 1:3. Finally,

the treatment protocol was found to strongly influence the resulting spectrum of eventual mutations, which implies that it may be possible to steer the evolution of resistance towards treatable adaptation.

Paper II

Rotating between ponatinib and imatinib temporarily increases the efficacy of imatinib in a cell line model

As predicted in Paper I, CML should not adapt as well to a drug rotation as it would to a monodrug protocol. In this work, we set out to test that prediction in CML cell lines. We chose KCL-22 and K562 for testing, both of which carry BCR-ABL1 and are sensitive to TKIs. The cells were exposed to drug rotations as well as monodrug protocols of imatinib and ponatinib over a six week period. Imatinib was chosen due to its status as the most prevalent CML TKI, whereas ponatinib was chosen specifically because it was predicted to perform well in drug rotations, see above. The experiments have already been described in great detail (Section 4.1).

In KCL-22 drug rotations performed slightly better than a monodrug protocol. Notably, growth was especially limited after the first exposure to ponatinib. It seems that imatinib performs better than normal when it follows ponatinib, though the mechanism which underlies this is unclear, and the effect was quickly lost with continued drug cycles. Eventually, the cells exposed to a drug rotation caught up with the growth of cells exposed to a monodrug protocol. K562 cells did not survive the experimental protocol; likely the combined stress of TKIs and the washing cycle proved too much for them.

The observed effect in KCL-22 hints at adaptations to ponatinib possibly sensitizing cells towards imatinib, as alternative explanations do not seem satisfactory. If the washing procedure could not eliminate enough ponatinib, leading to some drug interactions in the following drug cycle, we should expect to see the same effect every time imatinib follows ponatinib. Possibly, ponatinib may permanently alter drug efflux transporter activity, as it is known to be an inhibitor of ABCB1 and ABCG2. In conclusion, the experiment suggests that initiating treatment with a potent TKI like ponatinib, followed by imatinib for maintenance may be a viable strategy, and that a full drug rotation may be unnecessary. Such an approach is also very cost efficient compared to using second or third generation TKI long term.

Paper III

The effects of combination treatments on drug resistance in chronic myeloid leukaemia: an evaluation of the tyrosine kinase inhibitors axitinib and asciminib [66]

Paper III focussed on two drugs of particular interest: axitinib and asciminib. Axitinib is primarily used in RCC for its effect as a VEGFR, tyrosine-protein kinase KIT (c-KIT) and platelet derived growth factor receptor (PDGFR) inhibitor. It has been found to inhibit T315I mutated BCR-ABL1 specifically, while being ineffective against most other mutants of BCR-ABL1, as well as the unmutated form [132,133]. Thus, we desired to ascertain whether axitinib combined with a first or second generation TKI could mitigate their tendency to trigger the evolution of a T315I mutated malignancy.

We scanned a range of possible means of administering axitinib and a first or second generation TKI simultaneously, trying to minimize the selective advantage of T315I. Unfortunately, no approach could consistently eliminate it. As T315I is an extremely potent resistance mutation against most TKIs, no reasonable use of axitinib could remove that selective advantage. It was possible to very briefly invert it with a dasatinib–axitinib combination, as dasatinib is quickly eliminated from the body. However, T315I was still highly advantageous (or detrimental, from the patients point of view) for most of the day.

The other drug, asciminib, is a very recent discovery and is what seems to be the first successful product of a long line of effort into producing drugs targeting the myristoyl pocket of BCR-ABL1 [43]. The goal of allosteric inhibitor development is to discover a drug that is unaffected by the resistance mutations many current TKIs have in common. Furthermore, an allosteric drug may perform well in combination with ATP–pocket TKIs owing to not having to compete for binding. As such, there is reason to believe that asciminib and currently available TKI may have synergistic interactions.

We examined drug combinations of asciminib and first through third generation BCR-ABL1 TKIs, using practical and theoretical applications of the CI. In general, results were promising. We confirmed experimentally that asciminib and ATP–pocket TKIs seem to interact non-exclusively, which in practice confers a mild synergy. However, some antagonistic bias of unknown origin was present in the experiments. Knowing that there was a non-exclusive interaction, we investigated what administration protocols would allow for the biggest dose reduction compared to a single inhibitor using numerical optimization techniques. In general, simultaneous administration and aiming for each drug to be responsible for about half the total inhibition provided a near optimal result. The only exceptions were combinations involving dasatinib, which due to dasatinib being quickly eliminated from the body benefit from a staggered dosing protocol, such

as taking one drug in the morning, and one in the evening. Asciminib-containing drug combinations were also found to be fairly insensitive to resistance mutations, with the possible exception of T315I. Most mutations offered only a tiny degree of resistance according to our theory, likely of no clinical significance. Thus, asciminib in combination with a first or second generation TKI can offer both dose reductions and reduced risk of mutation-based resistance.

In this project we also compiled a, possibly comprehensive, dataset of known IC_{50} values for BCR-ABL1 mutants. In retrospect, it would have proven very useful in Paper I. Regardless, it may be of use to anyone modelling drug effects on BCR-ABL1 mutants in the future.

Paper IV

Modelling resistance in leukaemia mediated by mutations and alternate mechanisms – their interactions and treatment-free periods (drug holidays)

There is an experimental observation in CML cell lines where cells cultured in the presence of an inhibitor initially grow resistant through overexpression of some protein. Increases of BCR-ABL1, ABCG2 and tyrosine-protein kinase LYN (LYN) have all been observed [68,134–136]. After some time, a BCR-ABL1 KD mutant emerges and takes over, becoming the most common cell type. When the mutant takes over the initial adaptation reverses and returns to baseline, more or less.

To investigate this phenomenon, we designed a model consisting of two interacting resistance mechanisms: One weak and gradual, happening upon every division to some extent, and one rare but potent. The rare and potent mechanism models mutations, whereas the weak and gradual models increased expression. Protein expression is mediated through several mechanisms and we argue that the interactions of several possible means of changing it results in an approximately continuous scale of effects. Moreover, growth rates were a function of the expression levels in each cell, with a higher level than normal being advantageous for mutation free cells under treatment.

The model was qualitatively parametrized to reproduce the experimental observations using a continuum approximation and ABC. Stochastic simulations of the behaviours of mutant cells in a large bulk of normal cells were then carried out.

We show that this model exhibits tumour drug addiction effects, i.e. it seems possible to reduce overall growth of malignant cells by removing treatment at the right stage of adaptation. This always came with an increased risk of mutation however. Moreover, we showed that this model is most likely to incur either preexisting mutants or mutants that occur late, after a period of treatment.

Mutation mediated resistance was less likely before adaptation through increased expression had taken hold.

The model in Paper IV was written as a birth death process, but the individual treatment of cells makes is very apt for a branching process model as well. The numerical results would be equivalent, but the choice made analytical reasoning easier; something to consider in similar models.

Paper V

Inferring time-dependent growth rates in cell cultures undergoing adaptation

We found a need to track a dynamically changing growth rate of suspension cells with simple non-disruptive methods. Already available tools generally do not treat growth rate as a time-dependent variable, which is not conducive towards studying cells adapting to new circumstances. That feature combined with relying on simple measurements like cell counting was a combination that did not exist. We used it as an analysis tool in Paper II.

For our model, we started from a logistic growth with a *time-dependent* growth rate $r(t)$

$$\frac{dN}{dt} = r(t)N\left(1 - \frac{N}{K}\right). \tag{5.1}$$

Note the similarity to Equation 2.3; the above equation can also be solved but it is more complicated. In addition, a customizable filter to simulate how counting results, i.e. tiny samples of only 200 cells or so were produced by sampling and dilution was needed to complete the model. Using ABC we then reversed Equation 5.1 and the counting model to produce a piecewise linear growth rate starting from cell counts taken via a haemocytometer or an equivalent automated counter. We show that the method seems able to identify both temporary and absolute changes in growth rate in some simple test systems of KCL-22 and K562 cells.

This paper provides a new tool useful in some special cases where the goal is to induce a change in growth rate, and regular methods for static cell culture parameters are unsuitable. It was used successfully in Paper II.

Additional unpublished work

The drug rotation simulations from Paper I were also attempted for drug rotations involving axitinib in preparation for Paper III. Overall, results were not encouraging. Axitinib is not effective against any BCR-ABL1 variant except T315I. Hence the axitinib periods allow for more growth, thus producing more mutations that

could result in resistance towards the other drug. For this reason, axitinib in drug rotations was not investigated further (though there is a clinical trial [112], which seems ill advised in light of this). We also attempted to find optimal drug treatment protocols using the simulation engine from Paper I and a genetic algorithm metaheuristic. However, difficulties in normalizing the effectiveness of different drugs created intractable biases towards some of them. A tendency of switching drug once after a few month-equivalents could be seen, but the genetic algorithm approach was not tested further. In retrospect it may have been possible using the improved normalization from Paper III.

Finally, we cross referenced ExAC [65] against all known resistance mutations in targeted therapies from COSMIC [53] and My Cancer Genome [137]. Very few of the supposed resistance mutations occurred in ExAC (Figure 5.1 on page 58). In particular it seemed that the only mutations occurring naturally were ones that activate the enzyme (and confer resistance through that mechanism). Alternatively, some mutations are likely mislabelled as resistance mutations from occurring by chance in a drug-resistant patient. Regardless, this points towards artificial selection pressure induced by the targeted therapies as causative in the evolution of malignancies to harbour these mutations. It also indicates that in these cases, assuming all patients have the same genetic starting point is reasonably safe.

Significance

We provided evidence using CML–specific data indicating that drug rotations may have some protective effect, showing also that ponatinib is markedly more useful than other TKIs in this context. This was subsequently confirmed by experiment to some degree, although a single drug switch may hold most the benefit rendering full drug rotation extraneous. Overall, while more research is needed, this thesis supports the possibility of using drug rotations to lower the risk of drug resistance in CML.

To achieve the above, we also developed a niche, but practical tool for monitoring cells that undergo some kind of adaptation.

We further disparage the effectiveness of axitinib used against anything other than T315I. Axitinib may be useful if practically every malignant cell carries the T315I mutation, but any other use seems unwise according to our results. Asciminib on the other hand is indeed as promising as is generally suggested; our contribution consists of noting how combinations of asciminib with available drugs generally results in a potent mixture with few to no known resistance mutations.

Finally, we introduce novel model including both a gradual resistance through increased expression of some proteins, and the frequently studies resistance mutations. It provides an explanation of certain cell line observations, and

reveals some possible consequences of taking a break from treatment. We also discovered that there looks to be a gap of low mutation risk between preexisting and acquired mutations. Thus, if sequencing for mutations is applied defensively, an early test and then waiting a significant amount of time before testing again may be a cost-effective plan.

Discussion

Modelling work always makes simplifying assumptions. In Paper I, only resistance borne of BCR-ABL1 KD mutation is considered, and that in a constant-size pool of CSCs. What about starting points that include preexisting mutations? What about other means of acquired resistance? The second question is to some degree addressed in Paper IV, but there the distinct relationships between TKIs and SNVs (Figure 2.2) are ignored. This type of limitations are inherent in modelling studies, and balancing the approximations made with insightful results and tractable models is as much a science as it is an art. Broken down by article, the main modelling assumptions and there consequences were:

I – The model here was designed to use detailed information about how different mutations are affected by drug choice, including also a fine–grained model of the relevant genome. I argue that it achieves that goal, however, the long term effect of drug rotation protocols in practice likely requires taking many more aspects of CML into account, such as alternate resistance mechanisms, and a more accurate structure of the dynamics of CSCs. Ultimately, there is reason to believe drug rotations should have some protective effect based on this study, but the magnitude of the effect is hard to determine.

III – This paper used pharmacokinetics and drug synergy models to evaluate the effects of drug combinations. Rather than modelling evolution directly, it was assumed that mutants with a fitness advantage would be problematic, and avoiding that would be preferable. This is non-controversial, although slightly simplifies the issue. Arguably, there may also have been an overreliance on assuming simple pharmacokinetics correlates precisely with drug effects and cell growth, but this should only slightly fudge the precision of the timings we suggest.

IV – In stark contrast to Paper I, here many details were eschewed to emphasize the focus on the gradual adaptation mechanism of interest. This came at the cost of accurate quantitative predictions of any sort, but also enabled learning of the qualitative effects of including both a weak gradual resistance adaptation and highly resistant mutations.

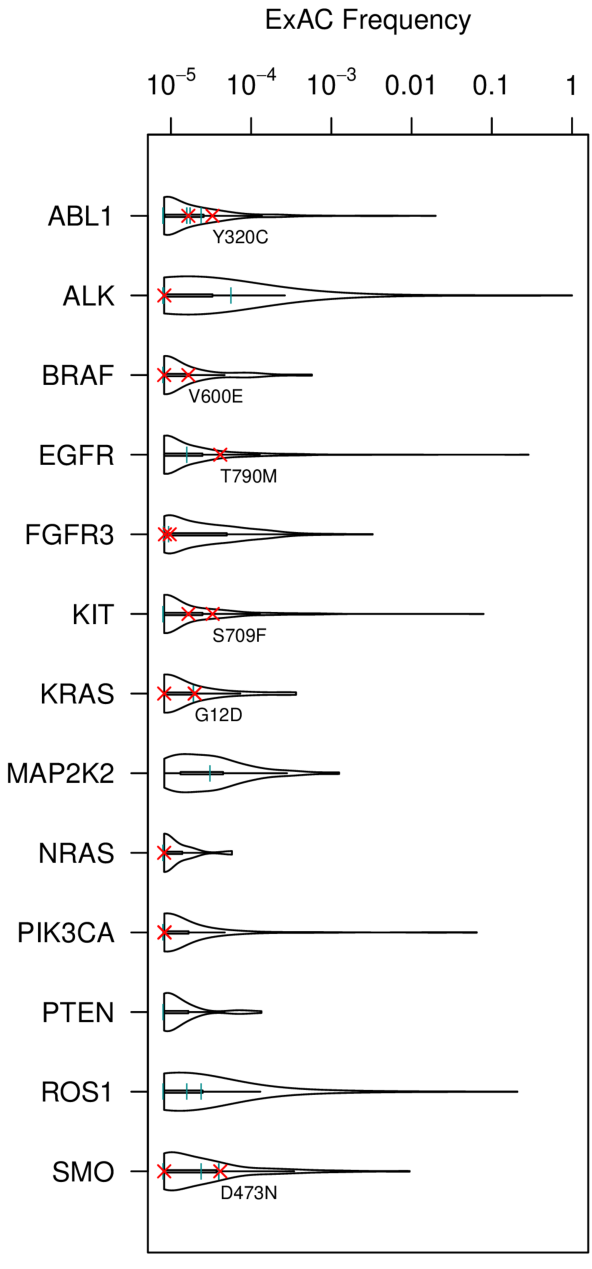

Figure 5.1: The occurrence of cancer drug resistance mutations in a healthy population. The violins show overall mutation frequency in that gene. The red crosses are exact matches between an alleged resistance mutation and a mutation found in the ExAC database. Common exact matches are labelled in the figure. The cyan lines are partial matches where the position matches but the amino acid substitution does not.

V – The methodology taken was very general, but leaves out the possibility of carrying capacity also changing. Doing so would however overdetermine the system, which would require some other assumption or new form of measurement to resolve.

On the experimental side, the drug synergy studies in Paper III are by the book, and it is unfortunate that the production of drug resistant cells to repeat the experiment in failed. If anything, it would be interesting to know whether extrapolating the three combinations tested to every TKI holds up in practice. The experiments of Paper II worked, but it seems improvements could have been made. For one, it may have been possible to run the experiment in K562 as well given some further tuning of inhibitor concentrations. Second, followup to learn the cause of observed effects is lacking, though a full investigation of the molecular biology was beyond the scope of this work. In conclusion, the experiments served their purpose providing support to the modelling studies, mostly confirming what was assumed and predicted.

Chapter 6

Conclusions and Future Work

6.1 Research findings

Most notably, there does seem to be non trivial treatment protocols that are better than what is currently being used. Drug rotations hold some objective potential, although the achievable effects are not massive. Ultimately, switching blindly could never outperform detailed sequencing for intelligent drug selection. However, drug rotations may double as a side effect management tool, which may be highly relevant, and they are also rather simple in their implementation. The possibility of tailoring drug rotations such that they steer evolution towards a treatable end point is intriguing, but the actual practice of this remains somewhat beyond our current capabilities.

The greatest advantages currently within our grasp seem to be asciminib-based drug combinations. The combination of synergistic interactions with a non-overlapping resistance mutation spectrum is very promising. Unsurprisingly, clinical trials of such are already under way.

The possibility of beneficial drug holidays are a curious option. From what we learned, it is unlikely that they could be used as a standard treatment modality. Notwithstanding, it is possible for a patient to be forced into taking a treatment break against their will or their doctor's better judgement. For instance

- TKIs are not recommended during pregnancy.
- The cost of some TKIs could make continuous treatment prohibitively expensive in some regions.

Thus, knowing the consequences of a drug holiday is valuable; even if it is not immediately exploitable for improved outcomes.

6.2 Future work

A better model of the internal hierarchic dynamics of CML CSCs is sorely needed. The dynamics of the evolution of resistance is greatly influenced by this. Possibly, there could be an answer within evolutionary graph theory [98], which can model evolution-suppressing systems in a natural manner. With a better internal model

of CSC dynamics, this research could in principle be repeated and refined to yield better predictions.

It would be very interesting to search for drug addiction effects in practice. A potential method would involve more accurate tracking of cell growth over time, coupled with high-frequency measurements of genotype and protein expression. I started construction of a morbidostat [138] for suspension cancer cells, but never finished due to the scale of the project, and having other priorities. Nevertheless, such a tool could answer many questions about the evolution of resistance.

There is still work to be done in elucidating the reason why drug rotations behaved as they did in KCL-22 cells. Experiments looking for each of the particular mechanisms capable of causing it would need to be designed. A first step would be sequencing for mutations, preferably at each intermediate step, and investigating possible residual drugs after washing.

Finally, revisiting the algorithmic optimization of treatment protocols is another appealing venture. With some work, the genetic algorithm may have been able to identify interesting and unexpected treatment protocols. After all, it is entirely possible that there may be hidden more severely non-trivial options available. In principle, such an algorithm could be seeded with a particular patients status, applying the techniques of personalized medicine.

Acknowledgments

I hope you have enjoyed reading my short book. There are some people I should mention, who have held pivotal roles throughout this endeavour.

First, I am grateful to my supervisor Ran Friedman for providing this opportunity. Thank you for steering my wild ideas towards the realms of reason, while still encouraging me to discover on my own.

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