RESEARCH ARTICLE

A Pharmacokinetic/Pharmacodynamic Model of the Action of Hypomethylating Agents in Chronic Myelomonocytic Leukaemia

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Abstract

Chronic myelomonocytic leukaemia (CMML) is a disease that borders between a myelodyplastic syndrome (MDS) and myeloproliferative neoplasia (MPN). It is a progressive condition: about 20% of CMML patients progress to secondary acute myeloid leukaemia (sAML), which has a very poor prognosis. In all cases, normal bone marrow function is compromised with resulting depletion of circulating erythrocytes and platelets. We modelled the dynamics of CMML at two levels: a cytokinetic model was developed that describes bone marrow cell population dynamics for multiple cell lineages, and changes in DNA mutational status that occur in MDS and CMML. This model, in conjunction with pharmacokinetic (PK) models for cytotoxic drugs and hypomethylating agents, was used to study the pharmacodynamics (PD) of these drugs. For the subset of CMML with loss of activity of TET2, our models suggest that TET2 activators may restore normal DNA methylation with a resulting antileukaemic effect. Mouse and human data were used to place bounds on the parameters of our model. Our modelling suggests that both hypomethylation and cytotoxic effects contribute to the antileukaemic activity of decitabine.

Keywords: Chronic myelomonocytic leukaemia, decitabine, DNA methylation, evolutionary dynamics, myelodysplastic syndrome, pharmacokinetic/pharmacodynamic model, TET2

Abbreviations used:
AML, acute myeloid leukemia; AUC, area under the plasma concentration-time curve; azaC, 5-azacytosine; azaC(d)R, 5-aza-2'-deoxycytidine; azaCR, 5-azacytidine; aza-CTP, 5-aza-2'-deoxycytidine 5'-triphosphate; CMML, chronic myelomonocytic leukemia; CNDAC, 2'-cyano-2'-deoxy-β-D-arabinofuranosylcytosine; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; HSC, hematopoietic stem cells; IV, intravenous; MDS, myelodysplastic syndrome; 5MeC, 5-methylcytosine; MPN, myeloproliferative neoplasia; PB-PK model, physiologically-based PK model; PD, pharmacodynamic(s); PK, pharmacokinetic(s); RBC, red blood cells (erythrocytes); shRNA, small hairpin RNA; TET2, ten-eleven translocation 2; TNF,

tumour necrosis factor; THU, tetrahydrouridine; WBC, circulating white blood cells;

1.0 Introduction

CMML is a heterogeneous group of diseases of ageing, and like other aspects of ageing, is characterised by epigenetic gene silencing. The heterogeneity of CMML can be largely explained by disordered stem cell or progenitor cell epigenetics[1,2]. The median age at onset of CMML is ~75 years of age, which is the highest of any cancer in Surveillance Epidemiology and End Results (SEER) data[1] and the median survival of CMML patients after diagnosis is 15 – 20 months. Approximately 20% of CMML patients progress to secondary AML (sAML), which has a very poor prognosis with a median overall survival of only ~6 months[2] and the remainder succumb to complications of bone marrow failure. The age distribution of CMML suggests that it is a disease caused by two (or more) hits. However, it is not highly genetically, or chromosomally, unstable, since only a small fraction of CMML patients progress to sAML. CMML shows elements of both hyperproliferation and dysplasia.[3] These features of CMML suggest that it may be the result of a single genetic mutation that results in hyperproliferation (e.g. a ras mutation) that, on its own, is not sufficient to cause transformation, combined with a second mutation (e.g. a TET2 mutation) that causes epigenetic instability.

The enzyme TET2 converts 5-methylcytosine (5mC) in DNA to 5-hydroxymethylcytosine (5hmC) and further oxidizes 5hmC to 5-formyl-cytosine (5fC), and 5fC to carboxylic acid cytosine (5caC). This causes 5mC conversions to C either actively by base excision repair of 5fC/5caC or passively by DNA methyltransferase 1 (DNMT1) not recognizing 5hmC after DNA replication[4] .The net result is re-activation of the epigenetically silenced gene. TET2 mutations associated with myeloid malignancies compromise catalytic activity, and result in DNA hypermethylation. Small hairpin RNA (sh-RNA) targeted against TET2 in mouse haematopoietic precursors skewed their differentiation towards monocyte/macrophage lineages in culture.[1] Approximately 50% of CMML patients carry TET2 mutations.[4] Other mutations commonly found in CMML include ASXL1 (40%), SRSF2 (40%), RUNX1 (20%), constitutively activated N-ras, JAK2 and mutant p53.[5-9]

Thomopoulos et al.[10], Chan et al.[11] and Kwon[12] have reviewed recent progress in CMML diagnosis and management. Increasingly, mutation analysis is being used to stratify the various subgroups of this heterogeneous disease[13,14]. Many of the mutations in CMML are associated with age-related changes in clonal haematopoiesis[15]. In addition to mutations in genes of epigenetic regulation such as TET2 and DNMT3A, mutations are often seen in ras genes and other components of the MAP kinase signalling pathway[16,17]. There are relatively few 'driver' mutations in CMML, which appears to result from genetic and epigenetic factors interacting with clonal haematopoiesis[18]. Both ancestral and subclonal mutations correlate with clinical features and prognosis of CMML[19]. The fact that CMML tends to have both epigenetic changes and constitutive MAP kinase signalling has been used as a rationale for combination treatment with decitabine and sorafenib[20].

Currently, CMML is often treated with the hypomethylating agents 5-azacytidine (azaCR) and 5-aza-2'-deoxycytidine (decitabine; azaCdR)[21] and in a study of CMML and MDS combined, response rates were higher in mutant TET2 cases.[22] This is consistent with CMML being a disease of abnormal DNA methylation, and with TET2 being a primary regulator of methylation patterns.

In previous studies[23,24] we used modelling

and simulation approaches to study the cellular pathology and evolutionary dynamics of chronic myeloid leukemia (CML). Unlike CML, which is at least initially a more homogeneous condition triggered by a chromosomal translocation, CMML is associated with a variety of mutations, but the fact that two FDA-approved agents for this disease, azaCR and azaC(d)R, are hypomethylating agents is consistent with epigenetic changes in gene expression being the primary driver of CMML. We model here the effects of azaC(d)R on the differentiation of myeloid progenitors to monocytes in an attempt to unravel the contributions of genetic and epigenetic changes to the aetiology of this disease.

2.0 Methods

2.1 SEER analysis

CMML was defined by the International Classification of Diseases for Oncology (ICD-03) code 9945. SEER analyses were performed using the R package SEERaBomb which is available from the Comprehensive R Archive Network (CRAN).

2.2 Disease modelling

The cytokinetic model CMMLsim is summarised in figure 1. It was modified from our published myelo.R model [23]. It consists of a set of ordinary differential equations describing the proliferation of cell populations in bone marrow, and their release into the circulation. Proliferation of the various cell lineages is stimulated by specific growth factors (erythropoietin, thrombopoietin, G-CSF, GM-CSF, interleukin-3). Under normal conditions, cell counts of the various lineages in the blood are maintained close to a set point by a feedback process which operates by the non-proliferating circulating cells binding the growth factors, and thus depleting the free growth factor levels available in the bone marrow (figure 2).

The mutations resulting in CMML may occur in HSC, in early-stage progenitor cells (myeloblasts) or in late-stage progenitors (promyelocytes). Note that in figure 2 the mutations are assumed to occur in late-stage progenitors, so there is no input to the dysplastic population from earlier-stage progenitors. The cytokines involved in monocyte proliferation, differentiation, and trafficking are summarised in figure 3. When modelling treatment of established CMML, a version of the model, CMMLhs4.R, was used in which the starting values of cell populations and cytokines were set to those previously calculated for two years after the mutations that triggered the condition.

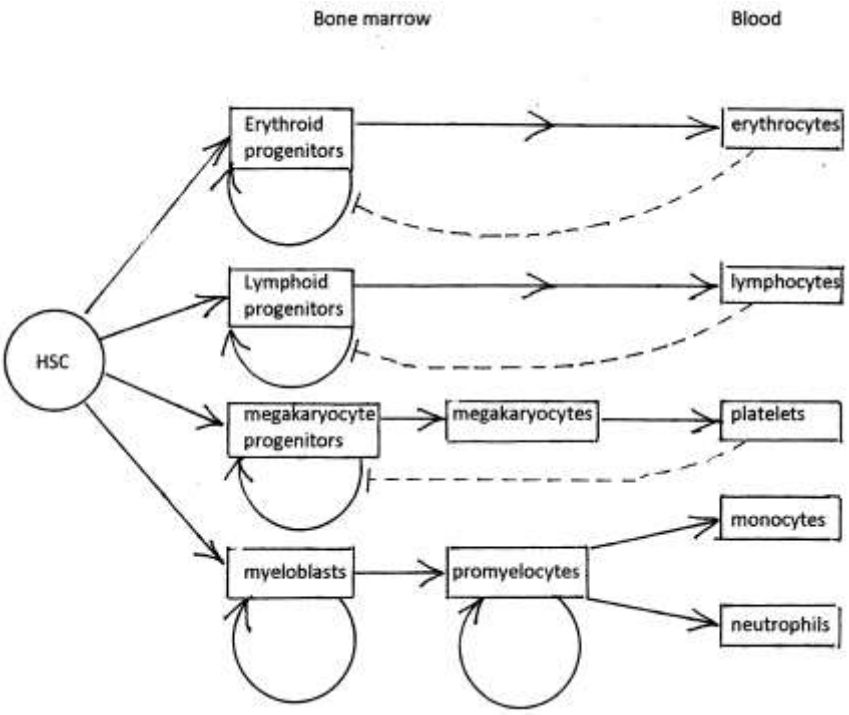

Figure 1. Cell populations described by the CMMLsim model. Haematopoietic stem cells (HSC) In bone marrow differentiate into four lineages. Progenitor cells in each lineage possess self-renewal capability, and also undergo further differentiation into non-proliferating end cells that are released into the blood.

2.3 PK/PD modelling

A schematic of the pharmacokinetic model PKmodel9 is shown in figure 4. It was adapted from the published Pkmodel8[25] by adding additional parameters to describe intracellular reactions of nucleoside- nucleotide interconversion and turnover. The PD model includes sites of action for twelve classes of drugs: cytotoxic agents, antagonists of GM-CSF, Bcr-Abl tyrosine kinase inhibitors, IL8 inhibitors, inhibitors of DNA replication, Mcl-1 transcription inhibitors, anti-TNF antibodies, STING agonists, alpha-interferon, antioxidants, macrophage activators, and hypomethylating agents. Two of these drug classes were modelled in the present study: hypomethylating agents (e.g. 5-azacytidine and decitabine) and DNA synthesis inhibitors (e.g. cytarabine, sapacitabine, CNDAC). The cytidine deaminase inhibitor, THU, was not explicitly modelled, but was treated as increasing the oral bioavailability of decitabine [26].

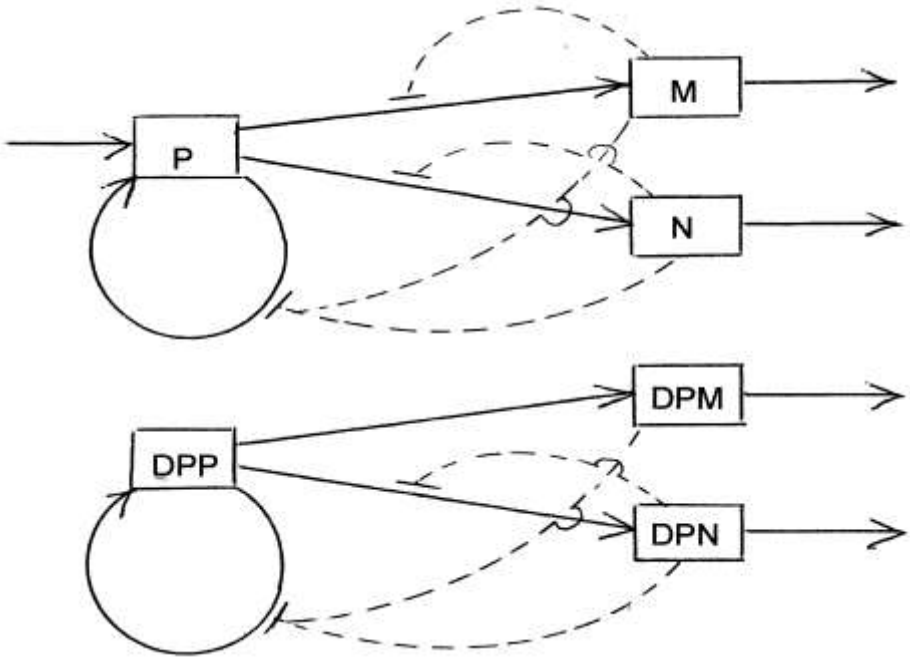

Figure 2. Regulation of the differentiation of myeloid precursor cells into monocytes and neutrophils, and its disruption in CMML. Feedback inhibition effects are shown with dashed lines. Upper panel: normal myeloid cells; lower panel: dysplastic cells. Note that the feedback inhibition of monocyte differentiation is missing in CMML. P = normal myeloid progenitors; M = normal monocytes; N = normal neutrophils; DPP = dysplastic progenitors; DPM = dysplastic monocytes; DPN = dysplastic neutrophils.

2.4 Fitting parameter values to the model

PK parameters for decitabine and for sapacitabine (a CNDAC prodrug) were obtained from the literature.[26-30] PKmodel9 was used to describe the PK of decitabine, sapacitabine, and the active metabolite of sapacitabine, CNDAC. The resulting concentration-time profiles were used as input for the CMMLsim cytokinetic simulations. Cytidine and its analogs have very poor oral absorption, because of high levels of cytidine deaminase in intestinal cells. The oral bioavailability of decitabine and other cytidine analogs is greatly increased by co-administration with the cytidine deaminase inhibitor, tetrahydrouridine (THU).[30-31]

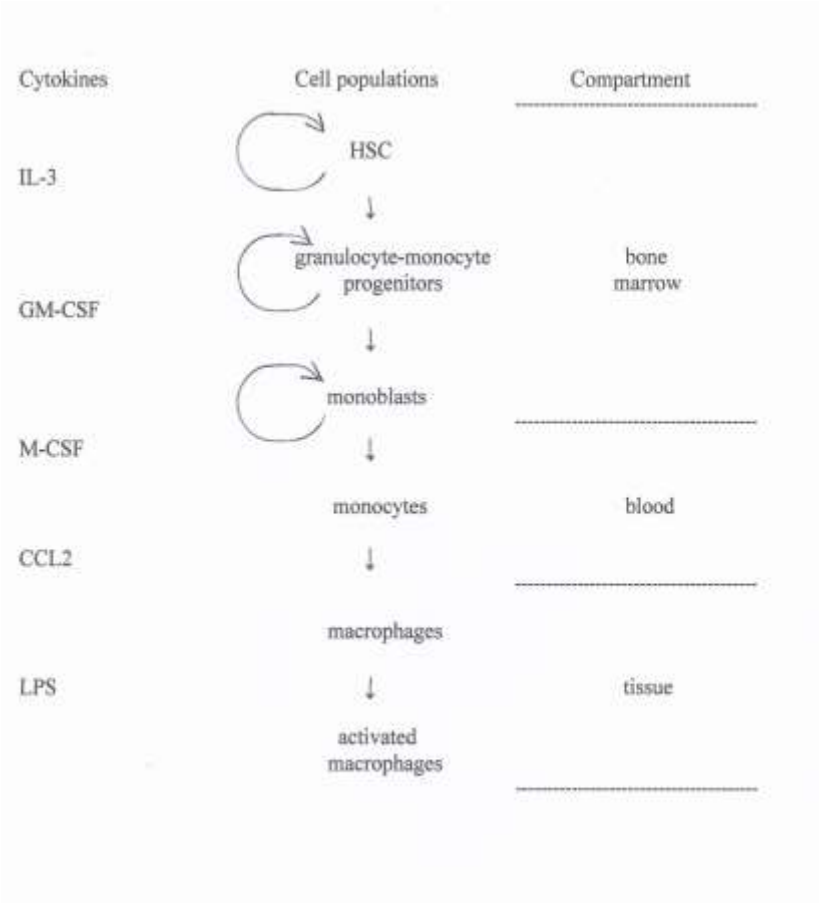

Figure 3. Cytokines involved in monocyte proliferation, differentiation and trafficking

Two pharmacodynamic endpoints were modelled. Negative regulation of monocytic differentiation is epigenetically regulated, and loss of this control (e.g. by TET2 mutation) thus causes excess monocytes. Hypomethylating agents then reduce the excess methylation and restore the negative regulation. The primary PD endpoint was thus the circulating monocyte count. Cytotoxic agents were assumed to inhibit proliferation of all replicating cell populations. Total WBC count was used as the secondary PD endpoint. PD parameter values for antipyrimidines were obtained from the literature.[32-35] Parameter values for intracellular metabolism of decitabine were based on data from HL60 leukaemia cells in culture, so must be regarded as approximate.

2.5 Programming

The computer implementation of our model, CMMLsim, is coded in the R programming language, and is available on GitHub. Systems of ordinary differential equations were solved using the R package deSolve, which is available from CRAN. A version of the model coded in C is also available. Plots were generated using R graphics.[36]

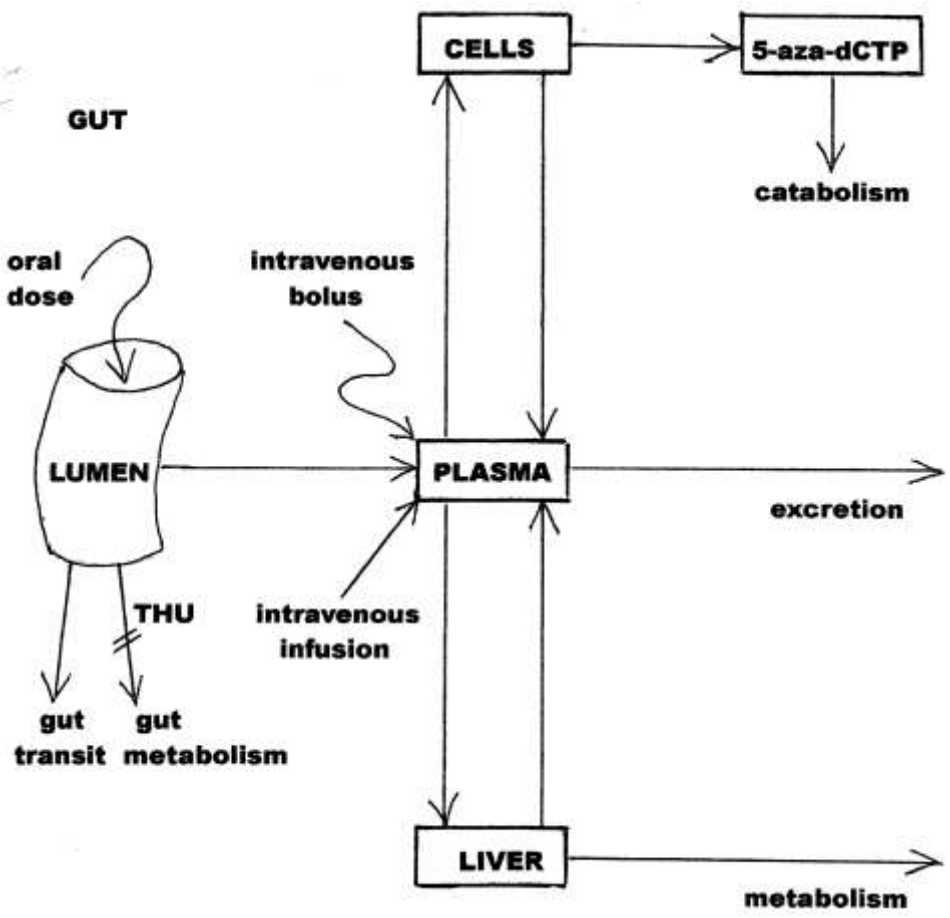

Figure 4. Compartmental analysis of the PK of 5-aza-2'-deoxycytidine, as described by the program Pkmodel9.

3.0 Results

The incidence of CMML with age, calculated from the National Cancer Institute's SEER database, is shown in figure 5. Up to the age of 50, the incidence is negligible, rising exponentially thereafter, with the suggestion that it may reach a plateau somewhere after the age of 90. Incidence at all ages is higher in men than in women.

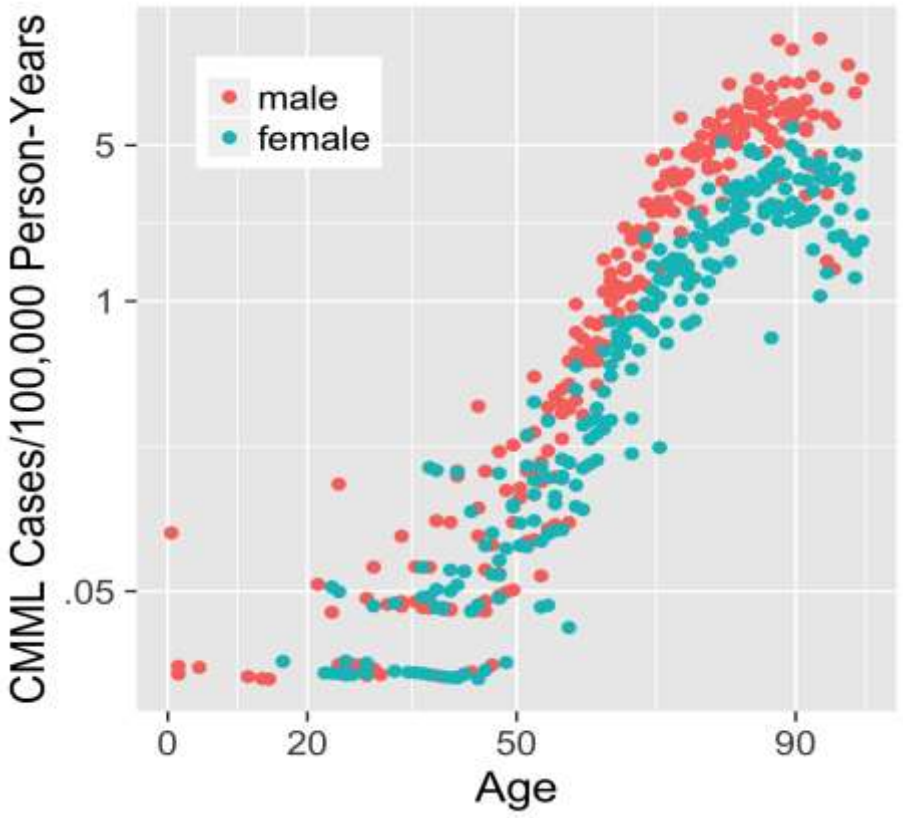

Figure 5. Age distribution of CMML, plotted from the NCI SEER database.

Output from the CMMLsim model, simulating the normal system in a steady state is shown in table 1. Note that table 1 shows only a small subset of the 27 state variables of the model. We then explored the potential effects of various mutations. These were assumed to occur either in haematopoietic stem cells (HSC), which rarely divide,[5] or in proliferating progenitor cells. The effect of an inactivating TET2 mutation, occurring in both alleles of a single HSC or myeloid progenitor cell TET2 -/-, on the population is modelled in table 2. Although the mutant cells were assumed to have the same growth rate as wild-type, the mutant cell was rapidly eliminated from the population (figure 6). In the absence of a compensating growth advantage, TET2 +/- progenitors were also competed out.

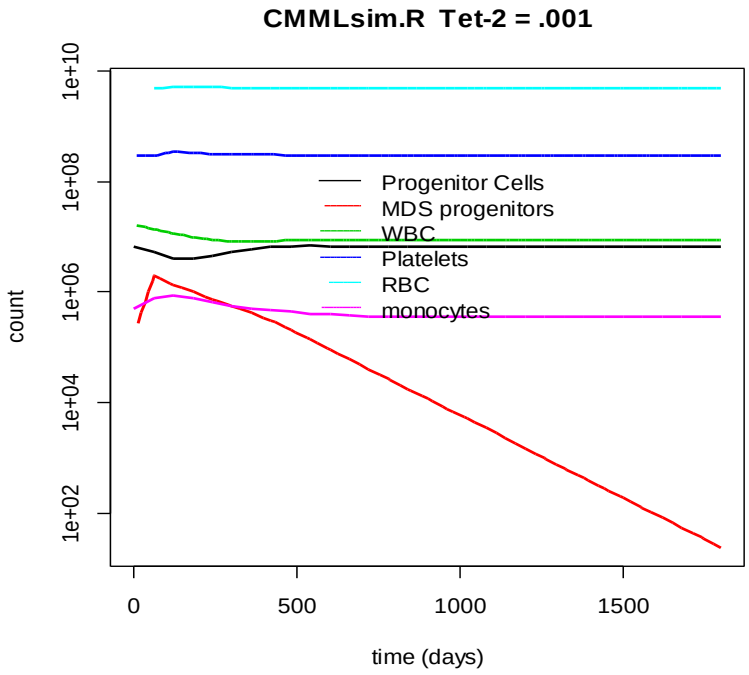

Figure 6. Modelling the consequences of a single cell with a TET2 mutation

N-ras mutations were assumed to stimulate cell growth, which they do by elevating cyclin D levels and thereby over-riding the G1:S checkpoint. HSC or progenitor cells with mutant ras can proliferate independently of GM-CSF, which frees them from feedback inhibition by circulating WBC. The effects of an N-ras mutation (again, assumed to occur in a single HSC or myeloid progenitor cell) are shown in table 3. There is a moderate (85%) increase in the total (normal+mutant) monocyte count, essentially no change in total WBC, and a concomitant decline in erythrocytes and platelets. This may be interpreted as hyperplasia of the myeloid population, but it does not meet the definition of CMML. Table 4 shows the calculated effect of an N-ras mutation followed by a mutation in a single TET2 allele. Over a period of about two years, the double mutant progenitors became the dominant marrow population, and the total circulating monocyte count increased about 2.5-fold. If, on the other hand, a TET2 mutation occurred first, the mutant clone became extinct before a ras mutation could occur, and CMML did not result. Modelling the situation of a clone of cells with mutant N-ras where a TET2 mutation in one allele was followed by a TET2 mutation in the other allele is summarised in table 5 and figure 7. The condition progressed from a single double-mutant cell to clinical disease in about 2 years, indicating that this combination of mutations had resulted in CMML. There were minor decreases in RBC and platelets, increased total WBC and a large increase in total monocytes (>15-fold), of which most were dysplastic

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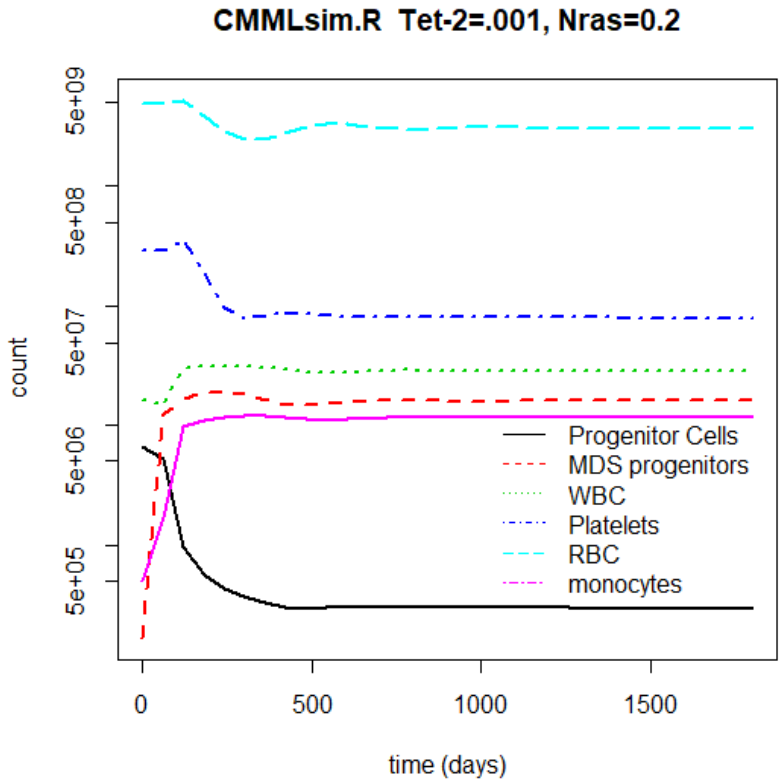

Figure 7. Progression from a singe double-mutant cell to clinical disease, modelled by CMMLsim.R

There is nothing specific about the effect of N-ras: constitutively activating ras mutations result in elevated cellular levels of cyclin D, over-riding the G1:S cell cycle checkpoint.[37] Other mutations that result in G1:S checkpoint over-ride, such as JAK2 mutations, are likely to have the same effect. The CMMLsim model has been used to study effects of mutant p53. Cells with mutant p53 have accelerated proliferation, because of G1 checkpoint override, but their proliferation is still GM-CSF-dependent. Like ras mutations or JAK2 mutations, p53 mutations are assumed to decrease the G1:S delay factor[37] but they do so by different mechanisms. Normal p53 stimulates transcription of p21 and p27, which act as inhibitors of cdk2/cyclin E and cdk4/cyclin D, required for cell cycle progression into S phase. Because of this mechanistic difference, ras mutations and p53 mutations have independent effects on G1:S progression delay (table 6).

Table 1. The uninhibited system as modelled by CMMLsim.

Time	P	WBC	Platelets	RBC	Monocytes	MKcytes	Neutro	Erythro
0	6.620e+006	8.457e+006	2.950e+008	4.820e+009	3.570e+005	1.030e+005	5.100e+006	1.600e+008
120	6.211e+006	8.386e+006	3.548e+008	5.128e+009	3.430e+005	1.084e+005	5.043e+006	1.641e+008
240	6.980e+006	8.492e+006	2.898e+008	4.857e+009	3.456e+005	1.006e+005	5.146e+006	1.564e+008
360	6.823e+006	8.682e+006	2.954e+008	4.788e+009	3.512e+005	1.045e+005	5.330e+006	1.580e+008
480	6.620e+006	8.610e+006	2.935e+008	4.811e+009	3.459e+005	1.031e+005	5.265e+006	1.600e+008
600	6.649e+006	8.552e+006	2.927e+008	4.827e+009	3.444e+005	1.032e+005	5.208e+006	1.597e+008
720	6.714e+006	8.572e+006	2.942e+008	4.820e+009	3.461e+005	1.036e+005	5.226e+006	1.591e+008
840	6.717e+006	8.595e+006	2.935e+008	4.815e+009	3.468e+005	1.033e+005	5.248e+006	1.590e+008
960	6.702e+006	8.595e+006	2.932e+008	4.815e+009	3.466e+005	1.033e+005	5.248e+006	1.592e+008
1080	6.700e+006	8.590e+006	2.933e+008	4.817e+009	3.464e+005	1.033e+005	5.244e+006	1.592e+008
1200	6.705e+006	8.591e+006	2.934e+008	4.817e+009	3.465e+005	1.033e+005	5.244e+006	1.592e+008
1320	6.707e+006	8.592e+006	2.933e+008	4.816e+009	3.466e+005	1.033e+005	5.246e+006	1.591e+008
1440	6.706e+006	8.593e+006	2.933e+008	4.816e+009	3.466e+005	1.033e+005	5.246e+006	1.592e+008
1560	6.705e+006	8.593e+006	2.933e+008	4.816e+009	3.466e+005	1.033e+005	5.246e+006	1.592e+008
1680	6.706e+006	8.593e+006	2.933e+008	4.816e+009	3.466e+005	1.033e+005	5.246e+006	1.592e+008

P, myeloid progenitor cells; WBC, total leucocytes; RBC, circulating erythrocytes; Mkcytes, bone marrow megakaryocytes; Neutro, circulating neutrophils; Erythro, bone marrow erythroblasts. Data shown as cells/L of blood or bone marrow.

Table 2. The effect of an inactivating TET2 mutation as modelled by CMMLsim

Time	P	MDP	WBC	Platelets	RBC	monocytes	MDM
0	7.036e+006	1.000e+000	5.725e+006	3.060e+008	4.920e+009	3.575e+005	7.000e-002
120	7.302e+006	4.724e-001	6.057e+006	3.552e+008	5.152e+009	3.715e+005	2.954e-001
240	7.557e+006	2.165e-001	5.988e+006	2.911e+008	4.888e+009	3.621e+005	1.925e-001
360	7.292e+006	9.250e-002	5.959e+006	3.064e+008	4.868e+009	3.603e+005	1.006e-001
480	7.204e+006	4.019e-002	5.861e+006	3.018e+008	4.893e+009	3.564e+005	4.756e-002
600	7.257e+006	1.783e-002	5.839e+006	3.019e+008	4.897e+009	3.566e+005	2.173e-002
720	7.284e+006	7.895e-003	5.859e+006	3.032e+008	4.891e+009	3.576e+005	9.792e-003
840	7.272e+006	3.477e-003	5.866e+006	3.024e+008	4.890e+009	3.576e+005	4.371e-003
960	7.262e+006	1.531e-003	5.861e+006	3.024e+008	4.891e+009	3.573e+005	1.939e-003
1080	7.264e+006	6.750e-004	5.859e+006	3.026e+008	4.892e+009	3.573e+005	8.574e-004
1200	7.267e+006	2.977e-004	5.860e+006	3.026e+008	4.892e+009	3.574e+005	3.787e-004
1320	7.267e+006	1.313e-004	5.860e+006	3.025e+008	4.891e+009	3.574e+005	1.672e-004
1440	7.266e+006	5.787e-005	5.860e+006	3.025e+008	4.891e+009	3.574e+005	7.374e-005
1560	7.266e+006	2.551e-005	5.860e+006	3.025e+008	4.891e+009	3.573e+005	3.252e-005
1680	7.266e+006	1.125e-005	5.860e+006	3.025e+008	4.891e+009	3.573e+005	1.434e-005

MDP, myelodysplastic progenitors in bone marrow; monocytes, total monocytes (normal + dysplastic); MDM, circulating dysplastic monocytes. Other abbreviations as defined in table 1. Data shown as cells/L of blood or bone marrow.

The simulations described in tables 1 – 6 have modelled the situation where one or more mutations have produced a single mutant progenitor cell, or a small clone of such cells, in the bone marrow. In the following simulations, the starting point was a post-diagnoses state of CMML, and the intent was to predict the pharmacodynamic effects of treatment. Initial PD simulations modelled treatment with the hypomethylating agent, decitabine. Table 7 shows the predicted effect of various steady-state plasma concentrations of decitabine. In agreement with clinical observation, over several weeks of treatment, decitabine caused

dose-dependent decline in the circulating monocyte count and in total WBC. The higher concentrations of decitabine, after prolonged treatment, reduced monocytes and total WBC below normal, probably reflecting the fact that in addition to its hypomethylating effect, decitabine is myelosuppressive. All concentrations of decitabine modelled increased the RBC count and the platelet count towards, but not above, their normal values.

Table 3. The effect of a constitutively active N-ras mutation as modelled by CMMLsim

Time	P	MDP	WBC	Platelets	RBC	monocytes	MDM
0	6.620e+006	1.000e+000	1.630e+007	2.950e+008	4.820e+009	3.570e+005	0.000e+000
120	4.237e+006	6.504e+000	1.003e+007	3.548e+008	5.128e+009	2.878e+005	1.385e-001
240	5.444e+006	3.216e+002	7.825e+006	3.155e+008	5.104e+009	2.859e+005	4.671e+000
360	7.123e+006	3.256e+004	8.212e+006	3.074e+008	4.905e+009	3.406e+005	4.230e+002
480	6.554e+006	1.583e+006	9.048e+006	2.923e+008	4.739e+009	3.755e+005	2.338e+004
600	2.312e+006	1.294e+007	1.346e+007	2.407e+008	4.329e+009	5.753e+005	3.334e+005
720	8.192e+005	1.587e+007	1.748e+007	1.401e+008	3.242e+009	6.721e+005	5.750e+005
840	5.030e+005	1.377e+007	1.691e+007	1.316e+008	3.217e+009	6.466e+005	6.035e+005
960	4.503e+005	1.368e+007	1.598e+007	1.306e+008	3.580e+009	6.317e+005	6.046e+005
1080	4.409e+005	1.479e+007	1.626e+007	1.262e+008	3.499e+009	6.543e+005	6.318e+005
1200	4.332e+005	1.485e+007	1.663e+007	1.235e+008	3.341e+009	6.645e+005	6.437e+005
1320	4.292e+005	1.449e+007	1.655e+007	1.222e+008	3.363e+009	6.593e+005	6.391e+005
1440	4.292e+005	1.450e+007	1.646e+007	1.211e+008	3.414e+009	6.574e+005	6.372e+005
1560	4.288e+005	1.466e+007	1.652e+007	1.207e+008	3.399e+009	6.606e+005	6.405e+005
1680	4.275e+005	1.466e+007	1.657e+007	1.206e+008	3.377e+009	6.621e+005	6.421e+005

Abbreviations as defined in tables 1 and 2. Data shows as cells/L of blood or bone marrow.

Table 4. The effect of an N-ras mutation followed by mutation of a single TET2 allele

Time	P	MDP	WBC	Platelets	RBC	monocytes	MDM
0	6.620e+006	1.000e+000	1.630e+007	2.950e+008	4.820e+009	3.570e+005	0.000e+000
120	4.237e+006	6.139e+000	1.003e+007	3.548e+008	5.128e+009	2.878e+005	2.400e-001
240	5.444e+006	2.849e+002	7.825e+006	3.155e+008	5.104e+009	2.859e+005	7.532e+000
360	7.125e+006	2.723e+004	8.212e+006	3.074e+008	4.905e+009	3.408e+005	6.511e+002
480	6.656e+006	1.288e+006	9.007e+006	2.926e+008	4.742e+009	3.847e+005	3.434e+004
600	2.602e+006	1.202e+007	1.298e+007	2.484e+008	4.403e+009	7.037e+005	4.711e+005
720	8.841e+005	1.573e+007	1.742e+007	1.470e+008	3.328e+009	9.109e+005	8.219e+005
840	5.280e+005	1.372e+007	1.704e+007	1.348e+008	3.232e+009	8.878e+005	8.510e+005
960	4.635e+005	1.345e+007	1.606e+007	1.346e+008	3.600e+009	8.646e+005	8.432e+005
1080	4.536e+005	1.456e+007	1.628e+007	1.305e+008	3.544e+009	8.947e+005	8.775e+005
1200	4.450e+005	1.470e+007	1.668e+007	1.279e+008	3.383e+009	9.113e+005	8.957e+005
1320	4.406e+005	1.433e+007	1.662e+007	1.264e+008	3.396e+009	9.048e+005	8.897e+005
1440	4.406e+005	1.432e+007	1.651e+007	1.254e+008	3.448e+009	9.015e+005	8.864e+005
1560	4.403e+005	1.448e+007	1.657e+007	1.250e+008	3.436e+009	9.058e+005	8.907e+005
1680	4.389e+005	1.449e+007	1.663e+007	1.249e+008	3.414e+009	9.081e+005	8.931e+005

Abbreviations as defined in tables 1 and 2. Data shows as cells/L of blood or bone marrow.

Table 5. The effect of an N-ras mutation followed by mutation of both TET2 alleles

Time	P	MDP	WBC	Platelets	RBC	monocytes	MDM
0	6.620e+006	1.000e+000	1.630e+007	2.950e+008	4.820e+009	3.570e+005	0.000e+000
120	4.237e+006	3.465e+000	1.003e+007	3.548e+008	5.128e+009	2.878e+005	8.224e-001
240	5.444e+006	9.209e+001	7.825e+006	3.155e+008	5.104e+009	2.860e+005	1.342e+001
360	7.133e+006	4.998e+003	8.209e+006	3.075e+008	4.905e+009	3.409e+005	7.444e+002
480	7.051e+006	1.487e+005	8.817e+006	2.939e+008	4.754e+009	3.789e+005	2.576e+004
600	5.725e+006	2.477e+006	9.608e+006	2.851e+008	4.745e+009	7.797e+005	5.125e+005
720	2.699e+006	9.594e+006	1.482e+007	2.453e+008	4.385e+009	3.566e+006	3.464e+006
840	1.356e+006	9.761e+006	1.746e+007	2.118e+008	3.998e+009	5.247e+006	5.216e+006
960	9.281e+005	8.605e+006	1.640e+007	2.249e+008	4.259e+009	5.087e+006	5.076e+006
1080	8.410e+005	9.318e+006	1.600e+007	2.281e+008	4.372e+009	5.074e+006	5.069e+006
1200	8.049e+005	9.854e+006	1.659e+007	2.289e+008	4.271e+009	5.377e+006	5.373e+006
1320	7.830e+005	9.610e+006	1.673e+007	2.273e+008	4.240e+009	5.438e+006	5.434e+006
1440	7.800e+005	9.481e+006	1.656e+007	2.265e+008	4.278e+009	5.365e+006	5.361e+006
1560	7.821e+005	9.592e+006	1.655e+007	2.270e+008	4.282e+009	5.368e+006	5.364e+006
1680	7.805e+005	9.633e+006	1.663e+007	2.272e+008	4.268e+009	5.402e+006	5.398e+006

Abbreviations as defined in tables 1 and 2. Data shows as cells/L of blood or bone marrow.

Table 6. Interaction of mutations in p53, N-ras and TET2

p53	N-ras	TET2	total monocytes	total WBC
normal	normal	1.000	3.48e+5	8.63e+6
normal	normal	0.500	3.51e+5	8.63e+6
normal	normal	0.001	3.48e+5	8.63e+6
normal	mutant	1.000	6.65e+6	1.67e+7
normal	mutant	0.500	9.12e+5	1.67e+7
normal	mutant	0.001	5.43e+6	1.67e+7
mutant	normal	1.000	7.38e+5	1.86e+7
mutant	normal	0.500	7.38e+5	1.86e+7
mutant	normal	0.001	6.65e+6	1.93e+7
mutant	mutant	1.000	1.02e+7	2.61e+7
mutant	mutant	0.500	1.41e+7	2.63e+7
mutant	mutant	0.001	1.16e+7	2.94e+7

Values of the G1:S checkpoint delay parameter: normal, 2.0; mutant N-ras, 0.5; mutant p53, 0.4; p53 and N-ras both mutated, 0.2.

Treatment with the DNA strand breaker, CNDAC is summarised in table 8. Like decitabine, this S phase-specific cytotoxic agent normalised the RBC and platelet counts, and decreased the total WBC and monocyte counts. The number of dysplastic progenitors in the bone marrow was sharply reduced at the higher concentrations. The pharmacodynamic effects CNDAC and the hypomethylating agent, decitabine, are mechanistically independent. If we define the IC50 of anti-CMML drugs as the concentration that reduces the monocyte count by 50%, then the IC50 of CNDAC, as calculated by the model is 85.7 nM, and the IC50 of decitabine is 28.6 nM. When we combined half the IC50 level of each drug, i.e.

42.85 nM CNDAC + 14.3 nM decitabine, the result was a decrease in the monocyte count of 52.2%. The combined effect is thus slightly greater than additive. In a more detailed analysis, we modelled the interaction of the CNDAC prodrug, sapacitabine, with decitabine. Results are shown in isobol form in figure 8; the isobol is slightly concave, indicating slight synergy. Analysis of the data by the method of Greco et al.[25] gave an alpha factor of 0.34; positive alpha factors indicate synergy.

Table 7. Treatment of CMML with decitabine.

Decitabine (μM)	RBC	Platelets	Total WBC	Monocytes
0	4.33e+9	4.54e+8	1.70e+7	5.64e+6
0.3	4.50e+9	4.75e+8	1.13e+7	8.62e+5
0.5	4.80e+9	5.13e+8	9.46e+6	6.07e+5
1.0	5.21e+9	5.66e+8	6.79e+6	3.46e+5
2.0	5.24e+9	5.70e+8	4.11e+6	1.28e+5
3.0	5.24e+9	5.70e+8	3.73e+6	9.47e+4

Cells/L of blood, as calculated by CMMLhs4.R. Values are steady-state levels after three months of treatment.

Table 8. Effect of plasma concentration of CNDAC on established CMML

CNDAC (μM)	RBC	Platelets	Total WBC	Monocytes
0	4.33e+9	4.54e+8	1.70e+7	5.64e+6
0.05	4.77e+9	5.10e+8	1.26e+7	3.65e+6
0.1	5.02e+9	5.42e+8	1.01e+7	2.55e+6
0.3	5.24e+9	5.70e+8	5.61e+6	6.87e+5
0.5	5.24e+9	5.70e+8	4.14e+6	1.52e+5
1.0	5.24e+9	5.70e+8	3.73e+6	9.46e+4

Cells/L of blood, as calculated by CMMLhs4.R. Values are steady-state levels after three months of treatment.

Modelling the effect of TET2 haploinsufficiency, i.e. the loss of activity of one of the TET2 alleles, indicated very little phenotypic effect. However, if the loss of one TET2 allele was preceded by a p53 mutation, the result was a kind of myelodysplasia, in which the RBC and platelet counts were depressed, total WBC were moderately increased (2.1-fold) and monocytes were 2.8-fold elevated. Treatment with decitabine had very little effect on this condition (table 9). At very high plasma concentrations there was a modest decrease in circulating monocytes, though this did not reach normalisation. Decitabine had no effect on the RBC or platelet count. Interestingly, CNDAC was active against this TET2 haploinsufficiency, restoring RBC and platelet counts to normal, and reducing the elevated total WBC and monocyte counts (table 10).

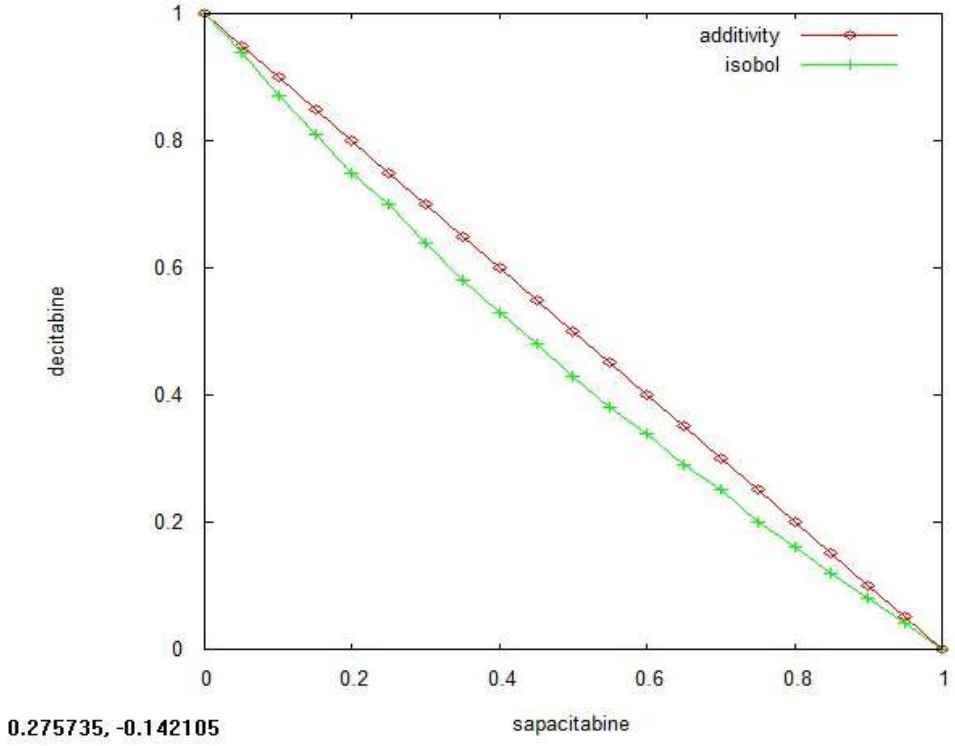

Figure 8. Isobol of the sapacitabine – decitabine interaction

Since decitabine acts both as a hypomethylating agent and as an S phase-specific cytotoxic agent, it is of interest to inquire which of the two effects is the primary cause of its therapeutic activity. This can be done computationally by removing one or other effect of decitabine from the model. Figure 9 shows dose-response curves for decitabine assuming either that both mechanisms are operative ("two targets") or that it is acting purely as a hypomethylating agent ("one target"). Hypomethylation alone resulted in an extensive decrease in the circulating monocyte count, but when the cytotoxic effect of decitabine was included in the model, its effect was greater, indicating that S-phase-specific cytotoxicity was also contributing to the antileukaemic effect of decitabine.

Table 9. Treatment of MDS resulting from TET2 haploinsufficiency with decitabine

Decitabine (μM)	RBC	Platelets	Total WBC	Monocytes
0	3.08e+9	8.34e+7	1.86e+7	1.01e+6
1.0	3.07e+9	8.34e+7	1.86e+7	1.01e+6
10.0	3.04e+9	7.95e+7	1.85e+7	7.70e+5
30.0	3.04e+9	7.92e+7	1.85e+7	7.48e+5

Cells/L of blood, as calculated by CMMLsim.R. The model assumes mutant p53 and TET2=0.5.Values are steady-state levels after three months of treatment.

Table 10. Treatment of MDS resulting from TET2 haploinsufficiency with CNDAC

CNDAC (μM)	RBC	Platelets	Total WBC	Monocytes
0	3.08e+9	8.34e+7	1.86e+7	1.01e+6
0.1	4.80e+9	2.91e+8	1.14e+7	3.04e+5
1.0	5.24e+9	3.45e+8	4.02e+6	1.16e+5

Cells/L of blood, as calculated by CMMLsim.R. The model assumes mutant p53 and TET2=0.5.Values are steady-state levels after three months of treatment.

Table 11. Effect of THU on oral bioavailability of 20 mg dose of decitabine

Treatment	AUC μmoles/L-min	Mean plasma level μM	Peak aza-dCTP μM	Time of peak (minutes)
IV infusion	176	0.120	0.443	182
oral	63	0.043	0.167	140
oral + THU	205	0.139	0.436	177

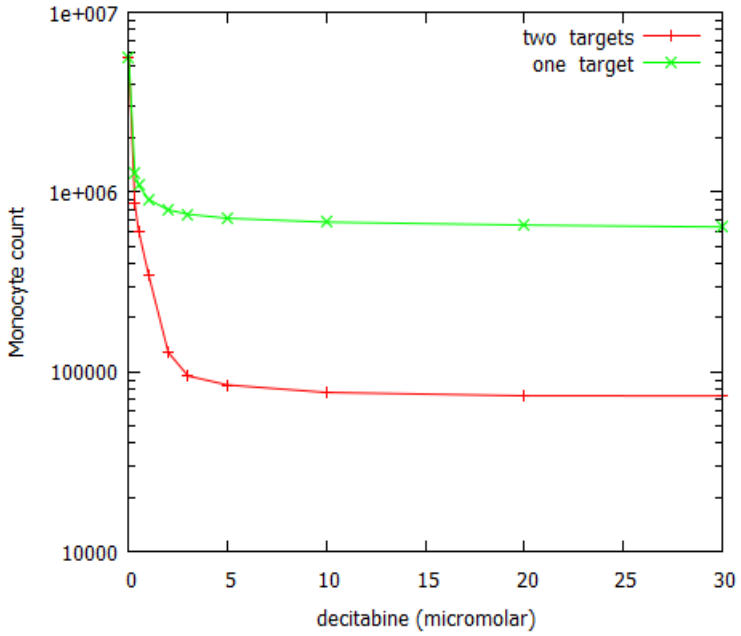

Figure 9. Dose-response curves for decitabine. One target: decitabine acts as a hypomethylating agent only; two targets: decitabine acts as a hypomethylating agent and an S-phase-specific cytotoxic agent.

Decitabine is usually administered by intravenous infusion. It is orally available, but enters the liver through the hepatic portal vein, and is rapidly deaminated by hepatic cytidine deaminase., so that its effective oral bioavailability is <10%. Co-administration of THU at 400mg/m^2 increased the oral bioavailability of decitabine to >80%.[30] Calculations with a PB-PK model (figures 10 – 12, table 11) predicted that co-administration of THU with oral decitabine resulted in AUC, mean plasma concentration, and intracellular deaza-CTP similar to levels obtained after IV infusion.

4.0 Discussion

Monocyte and macrophage differentiation and activation are known to be epigenetically regulated [38] and mutations in TET2, a key epigenetic regulator, are among the commonest genetic abnormalites in CMML.[15,39] Having a model of multiple cell populations in bone marrow, their proliferation, differentiation, and mutual competition, made possible prediction of the effects of mutations, singly and in combination. The model predicted that a single replicating bone marrow cell with a TET2 -/- mutation, or a small number of such cells, would become extinct, despite the fact that the dysplastic progenitors were assumed to have the same growth rate as wild-type myeloid progenitor cells (figure 6). The hypermethylation resulting from the TET2 mutation disrupted feedback inhibition of monocytic differentiation. The greater rate of differentiation of dysplastic progenitors disrupted the balance between their proliferation and release into the circulation, so that they were out-competed by normal myeloid progenitors, and rapidly disappeared.

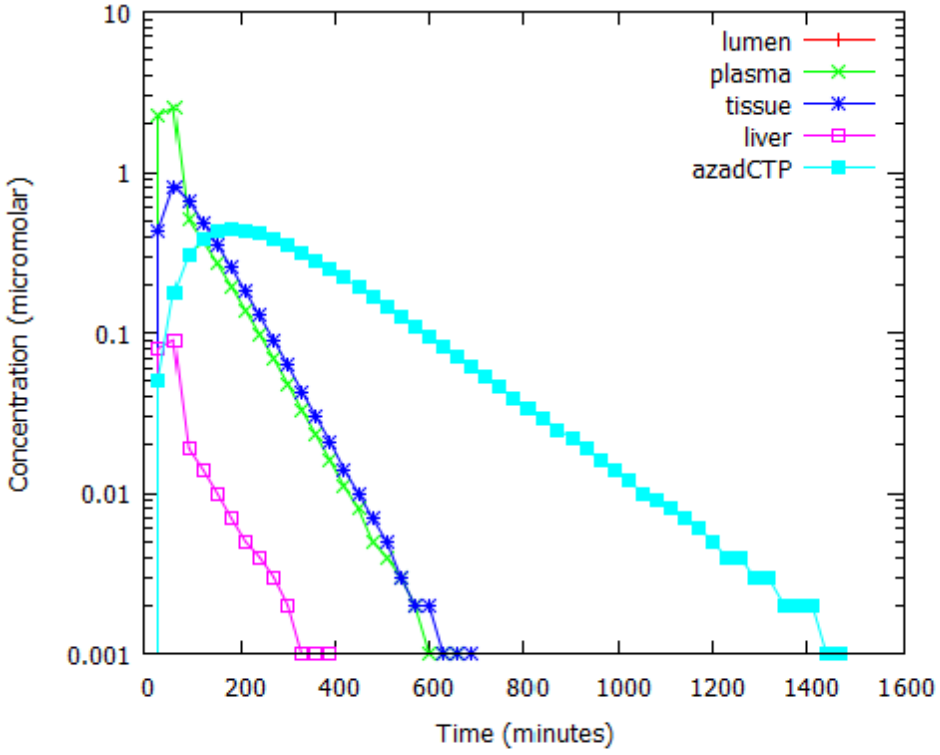

Figure 10. Blood and tissue levels of decitabine following a 1-hour infusion of 20 mg, calculated by Pkmodel9.

An N-ras mutation caused an approximate 2-4-fold increase in the circulating monocyte count and total white cell count, and a modest decrease in the count of red blood cells and platelets. As with epithelial tissues, ras mutations in the absence of other mutations cause a nonmalignant hyperplasia. However, a ras mutation followed by a TET2 mutation resulted in CMML, with a large increase in the circulating monocyte count and WBC count. Note that increased self-renewal of the dysplastic progenitors suppressed the normal progenitors (table 5) as reported experimentally.[40] The same combination of mutations in the opposite order would be unlikely to occur, since a TET2 mutated clone would, according to the model, become extinct before a second mutation could occur. The cooperative effect of N-ras and TET2 mutations in driving myeloid cell transformation has been described experimentally,[6] and our model suggests a possible mechanism for this effect.

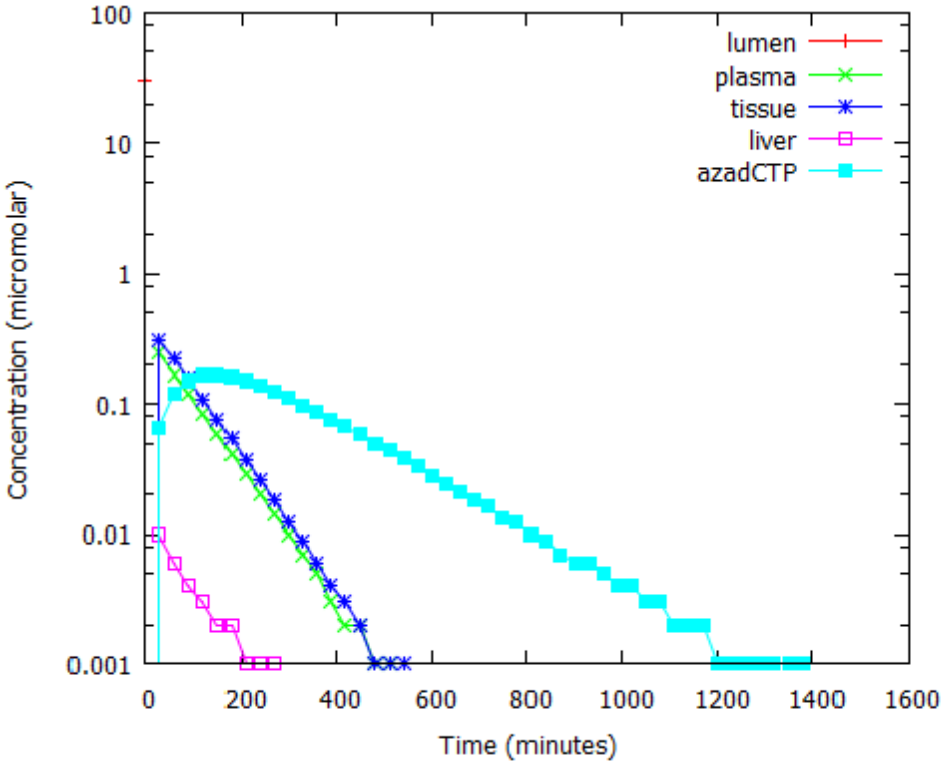

Figure 11. Blood and tissue levels of decitabine following a 20mg oral dose of decitabine

Since loss of TET2 activity is clearly a causative factor in CMML, it seems likely that TET2 activators, if such can be identified, might be effective treatments for CMML. Increasing ascorbate levels is a possible approach. The other TET2 substrate, α-ketoglutarate, is probably too rapidly metabolised in the tricarboxylic acid (TCA) cycle for this to be a practical approach, but analogs of α-ketoglutarate that are not substrates for the TCA cycle are a possibility.

Can hypomethylating agents be used to restore bone marrow homeostasis? To prevent or delay disease progression? Existing hypomethylating agents, 5-azacytidine and decitabine decrease DNA methylation following their incorporation into DNA. Both suffer from the disadvantage that they are

substrates for cytidine deaminase, particularly if it is administered orally. Coadministration of THU is an established method of increasing the oral bioavailability of decitabine[26] and probably increases its incorporation into DNA. Our model suggests that cellular aza-dCTP levels remain high for several hours after treatment; however, the important endpoint is DNA incorporation, which we are at present unable to model. Once incorporated, azaC will remain in DNA until the cell dies, though it will be progressively diluted out as a result of DNA replication.

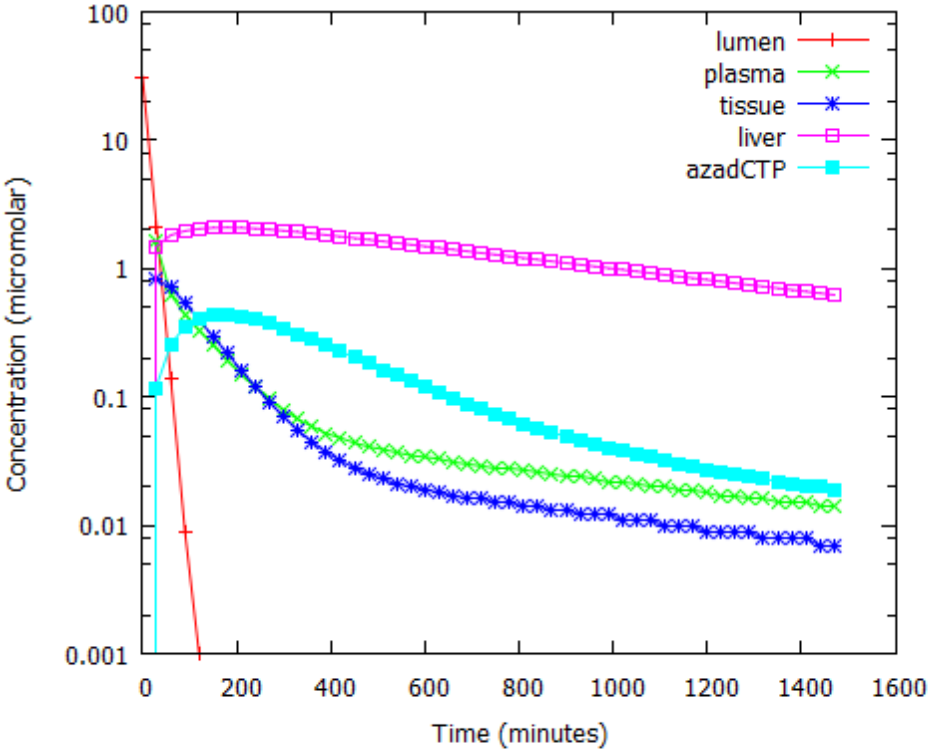

Figure 12. Blood and tissue levels of decitabine following a 20mg oral dose of decitabine co-administered with 400 mh THU

Our cytokinetic/pharmacodynamic models of CML suggested ways in which the treatment of that disease might be optimised to delay progression of CML to its terminal blast crisis stage.[23,24] CMML has both similarities and differences from CML: like CML, CMML is associated with hyperproliferation of myeloid precursor cells, and like CML it sometimes progresses to a form of acute myeloid leukaemia (AML). Modelling CMML could deepen our understanding of the disease process, and may suggest ways to optimise its treatment.[41]

Is CMML a malignant disease, or a premalignant condition? If we regard genetic instability as the essential hallmark of malignancy, then the 20% of CMML cases that progress to sAML are unquestionably malignant. The other 80% die of other morbidities; if this were not the case, would they, too, have progressed to sAML? It would be interesting to know if the mutation profile of the 20% that progress is different from that of the 80% that do not.

There is an apparent contradiction between the prediction of our model, that TET2

loss or haploinsufficiency, in the absence of an additional mutation that provides a growth stimulus, made the mutant progenitors non-viable, and the report[42] that in a mouse model of TET2 loss, the self-renewal capacity of myeloid precursors was increased. This difference may be explained by our simplifying assumption (in the model) that TET2 loss only affects monocytic differentiation. In fact, the effects of TET2 loss are likely to be pleiotropic, and DNA methylation may be a negative regulator of myeloid progenitor self-renewal. Nevertheless, the fact that growth-stimulatory mutations are so common in CMML (N-ras, p53, JAK2) suggests that some cases of human CMML, at least, require an additional growth stimulus for transformation.

In summary, we propose that CMML is a three-hit malignancy, requiring minimally (a) a growth-promoting mutation (or possibly loss of epigenetic silencing of a negative growth regulator); (b) increased expression of a normally silenced regulator of monocyte differentiation, often by loss of a single TET2 allele; and (c) loss of the second TET2 allele, resulting in increased methylation, and consequent silencing, of an enhancer region controlling the negative regulation of monocyte differentiation. The age distribution of CMML is strong evidence for the multi-step origin of this leukaemia.

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