A Computational Model of Thiopurine Metabolism

Sarah McGarrity

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Abstract

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The thiopurines, azathioprine, mercaptopurine (MP) and thioguanine are used as immunosuppressants and in the treatment of leukaemia. These drugs undergo extensive metabolism to form their cytotoxic metabolites that correlate with drug efficacy and the likelihood of side effects. Although these inexpensive drugs are effective in many patients, a deeper understanding of thiopurine metabolism would enable better individualisation of therapy resulting in increased efficacy and safety.

A computational model of MP metabolism using data generated from mercaptopurine treated MOLT-4 (human T-ALL cell line) by a novel liquid chromatography mass spectrometry assay was built in CoPaSi. The model qualitatively reproduced published data about the effects of changes in thiopurine methyltransferase activity on MP metabolism.

In vitro studies showed that high concentrations of allopurinol, a xanthine oxidase inhibitor, reduced the sensitivity of MOLT-4 cells to MP from 2.9 µM to 43 µM ($P<0.001$), whereas lower concentrations of allopurinol slightly but not significantly increased the sensitivity of MOLT-4 cells to MP.

Combination of MP and allopurinol treatment of MOLT-4 cells resulted in lower concentrations of thioguanine nucleotides and methylated thioinosine monophosphate metabolites compared to MP only treatment, as determined by liquid chromatography mass spectrometry.

These data were used to extend the model of MP metabolism to test hypotheses that addition of allopurinol decreased methylated thioinosine monophosphate and increased the concentration of TGNs. The computational model suggested that the mechanism by which allopurinol interacts with MP metabolism is by inhibiting hypoxanthine guanine phosphoribosyl transferase resulting in altered levels of MeTIMP and TGNs.

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Dr Graham Smith assisted with using a python tool for model translation to SBMLshorthand and back to SBML described in Chapter 4. I would like to thank him for his help with this and the systems modelling group more generally for their comments at our group meetings.

Dr Svante Vikingsson introduced me to chromatography and provided a starting point to develop a high pressure liquid chromatography method for thiopurine detection described in Chapter 3.

Mr Philip Berry suggested that a LC-MS method would be more sensitive and assisted in setting up the method described in Chapter 3, especially with the initial tuning of metabolites and selecting appropriate buffers. I would also like to thank him for all his support during this project.

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Abbreviations

adenosine triphosphate - ATP

azathioprine – AZA

bicinchoninic acid - BCA

concentrative nucleoside transporter - CNT

de novo purine synthesis - DNPS

dithiothreitol - DTT

electro spray ionisation - ESI

equilabrative nucleoside transporter - ENT

erythrocytes - RBC

ethylenediaminetetraacetic acid - EDTA

eXtensible mark-up language - XML

Food and Drug Administration - FDA

generalised mass action - GMA

glutathione-s-transferase - GST

graphical user interface - GUI

guanosine monophosphate synthase - GMPS

guanosine triphosphate - GTP

high pressure liquid chromatography - HPLC

homologous recombination - HR

hypoxanthine – HX

hypoxanthine guanosine phosphoribosyl transferase - HGPRT

inflammatory bowel disease - IBD

inosine monophosphate dehydrogenase - IMPDH

inosine tri phosphate - ITP

inosine tri phosphate phosphatase - ITPA

liquid chromatography mass spectrometry – LC-MS/MS

mass spectrometry - MS

mercaptopurine - MP

methyl mercaptopurine – me MP

methyl mercaptopurine riboside – me MPR

methyl tetrazolium sulphate – MTS

methyl thioadenosine phosphorylase – MTAP

methyl thioguanosine diphosphate - meTGDP

methyl thioguanosine monophosphate – meTGMP

methyl thioguanosine triphosphate – meTGTP

methyl thioinosine diphosphate - meTIDP

methyl thioinosine monophosphate – meTIMP

methyl thioinosine triphosphate - meTITP

mismatch repair – MMR

multi-drug resistance protein 4– MRP 4

nicotinamide adenine dinucleotide – NAD

ordinary differential equation - ODE

peripheral blood mononucleocytes – PBMC

phosphate buffered saline – PBS

phosphoribosyl pyrophosphate – PRPP

phosphoribosyl pyrophosphate synthase – PRPPS

quality control – QC

reactive oxygen species – ROS

standard deviation - SD

systems biology graphical notation – SBGN

systems biology mark-up language – SBML

tandem mass spectrometry – MS/MS

thioguanosine diphosphate – TGDP

thioguanosine monophosphate – TGMP

thioguanosine nucleotides – TGN

thioguanosine triphosphate – TGTP

thioinosine monophosphate – TIMP

thioinosine triphosphate – TITP

thiouric acid – TUA

thioxanthine – TX

thioxanthosine monophosphate – TXMP

thioguanine – TG

thiopurine methyl transferase – TPMT

uric acid – UA

xanthine oxidase – XO

xanthosine monophosphate – XMP

Chapter 1. Introduction to thiopurines and systems biology

1.1 Introduction

Thiopurines were developed by Elion and Hitchings during the 1940's and '50's (Elion *et al.*, 1988). Following observations that endogenous purines were essential to cell replication it was hoped purine analogues would interfere with endogenous purine metabolism and would lead to a reduction in replication of cancer cells (Elion *et al.*, 1988). The thiopurines mercaptopurine (MP), azathioprine (AZA) and thioguanine (TG), are now used to treat paediatric leukaemia (Clinical Trial Service Unit) and autoimmune diseases such as inflammatory bowel disease (IBD) (Gleeson and Heneghan, 2011; Girardin *et al.*, 2012; Bressler *et al.*, 2015) and as immunosuppression after transplantation (Scheffert and Raza, 2014). They are pro-drugs which rely on a complex metabolism to produce their active metabolites; thioguanosine nucleotides (TGNs) incorporated into DNA and the thioguanosine mono, di and tri phosphates (TGMP, TGDP and TGTP). This metabolism shows variation between individuals, as does the efficacy.

There is a variation in the efficacy of thiopurine treatment between patients; this is due to the variation in production of thiopurine metabolites. For example around 30% of Crohn's Disease patients, a type of IBD (Cuffari *et al.*, 1996b), do not respond to treatment with thiopurines, and this often corresponds with a low level of TGNs (Cuffari *et al.*, 1996b; Wright *et al.*, 2004; Osterman *et al.*, 2006). Other patients treated with thiopurines produce too many TGNs, frequently due to lack of the methylating enzyme thiopurine methyltransferase (TPMT) (Enzyme Commission (E.C.) 2.1.1.67), and experience complications including neutropenia, leukopenia and pancytopenia (Lennard *et al.*, 1997). A further group of patients produce excessive amounts of methylated thiopurine metabolites, products of an alternative pathway of metabolism, and these have been linked to hepatotoxicity (Dubinsky *et al.*, 2000; Derijks *et al.*, 2004; Gardiner *et al.*, 2008b). TGN concentration has also been shown to be important for predicting prognosis in leukaemia after MP treatment (Schmiegelow *et al.*, 1995).

Many clinical studies have found evidence of correlations between levels of thiopurine metabolites, which are linked to treatment efficacy, and the activities

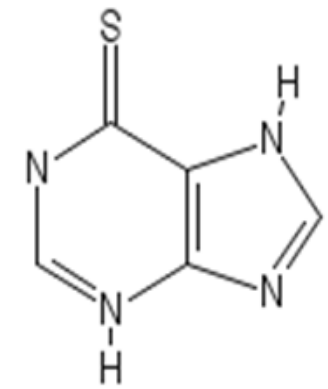
and expression of various enzymes, mostly those involved in endogenous purine metabolism. There have also been cell-based studies seeking to provide more detailed mechanistic insights into variation in the production and effects of thiopurine metabolites.

Systems biology approaches biological questions by examining whole systems in order to understand their structure and dynamics. This is in contrast to traditional biological approaches which focus in detail on individual components (Kitano, 2002b). One technique of systems biology is using detailed information on molecular mechanisms to build computational models (Wolkenhauer, 2014). This approach can enable an improved understanding of the relationships between system components and their emergent behaviours.

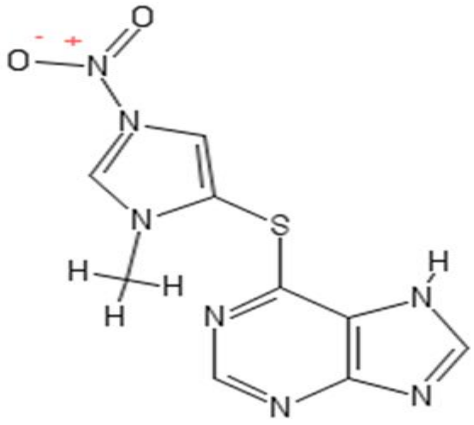
The metabolism of thiopurines is a complicated but well-studied system. Despite over 60 years of investigation it is still impossible to predict fully which patients will benefit from what dose of thiopurine and in what combination with other drugs. It was envisaged that creating a computational dynamic model would allow insights to be gained about the combined effects of the many components of the metabolism of thiopurines that have previously been studied.

1.1.2 Thiopurine structure

The thiopurines are sulphur-substituted analogues to endogenous purines. AZA (6-(3-methyl-5-nitroimidazol-4-yl)sulfanyl-7H-purine) and MP (3,7-dihydropurine-6-thione) are analogues of hypoxanthine (HX) and TG (2-amino-3,7-dihydropurine-6-thione) is an analogue of guanine (Figure 1.1 a and c). In all three drugs, the oxygen normally bonded to the 6-carbon atom is replaced by sulphur as indicated by the chemical names (Van Scoik *et al.*, 1985). In addition AZA has an imidazole moiety attached to this sulphur (Figure 1.1b). AZA was developed shortly after MP. It was thought that the extra reaction removing the imidazole group from MP would allow for a steadier production of active metabolites over time. Early studies indicated that the addition of the imidazole group increased the therapeutic index of MP.



a



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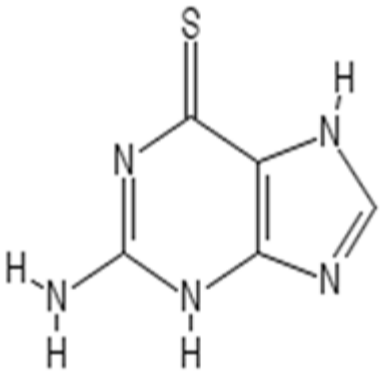


c

Figure 1.1 The chemical structure of **a** MP **b** AZA, showing the sulphur bonded to the position 6 carbon and **c** TG drawn in PubChem Sketcher version 2.4 ((*https://pubchem.ncbi.nlm.nih.gov/edit2/index.html*))

1.1.3 Thiopurine metabolism

The metabolism of thiopurines is a multi-step process that mirrors the reactions of endogenous purine recycling (Figure 1.2).

AZA is first metabolised to MP by removal of the imidazole group by reaction with glutathione normally catalysed by glutathione-s-transferase (GST) (E.C. 2.5.1.13), although around 1% of the reaction of glutathione and AZA is non-enzymatic. There are various subtypes of GST, A1-1, A2-2 and M1-1 that are abundantly expressed in liver and highly active with respect to AZA. Homozygosity for the GST-M1 deletion is found in around 50% of the population. This mutation has been shown to reduce the efficacy of AZA by reducing the amount of TGNs produced (Stocco *et al.*, 2014). Other allelic variants of GST have increased activity and can lead to adverse drug reactions (Zhang *et al.*, 2010).

MP and TG are both taken up into cell types by concentrative nucleoside transporters (CNT) 1,2 and 3 (Peng *et al.*, 2008) and equilbrative nucleoside transporter 2 (ENT) (Fotoohi *et al.*, 2006a; Nagai *et al.*, 2007). MP may also be removed by multi-drug resistance protein 4 (MRP4) (Krishnamurthy *et al.*, 2008). The expression levels of these proteins have been shown to correlate with sensitivity of cells to thiopurines.

MP and TG are both metabolised by hypoxanthine-guanine phosphoribosyl transferase (HGPRT, E.C. 2.4.2.8) to form their respective mono phosphate nucleosides, thioinosine monophosphate (TIMP) and TGMP, by addition of ribose sugar and a phosphate group. Cells which lack HGPRT are resistant to thiopurine drugs (Brockman *et al.*, 1960). However normal variation in activity of HGPRT does not appear to determine differences in MP response (Pieters *et al.*, 1992; Lennard *et al.*, 1993).

TIMP is further metabolised to produce thioxanthosine monophosphate (TXMP) by nicotinamide adenine dinucleotide (NAD) dependent oxidation by inosine monophosphate dehydrogenase (IMPDH, E.C. 1.1.1.205). IMPDH activity has been observed to correlate positively with methyl thioinosine monophosphate (meTIMP)/TGN ratio (Haglund *et al.*, 2011). IMPDH activity has been shown to increase *in vitro* after TG treatment (Vethe *et al.*, 2008), possibly due to increased demand for endogenous guanosine nucleotides.

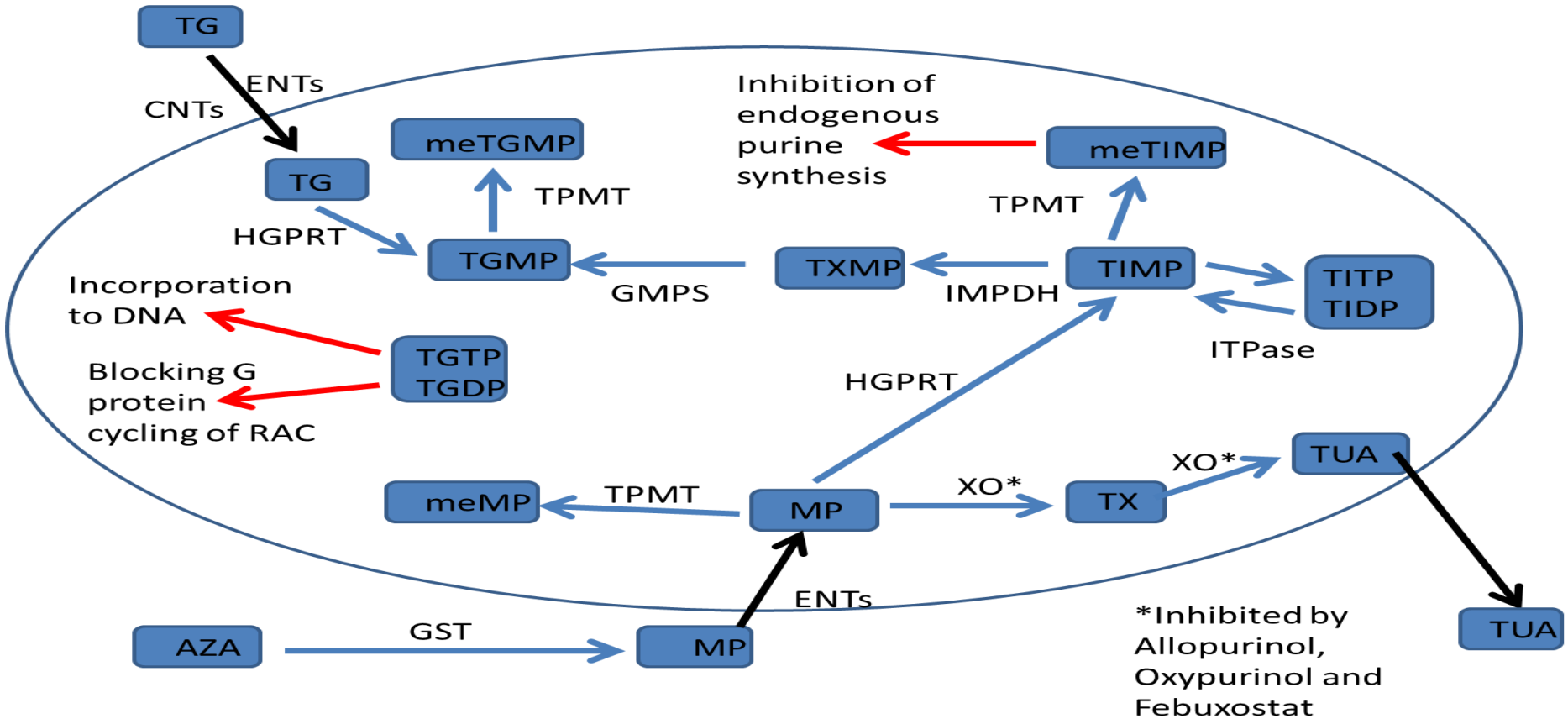


Figure 1.2 The metabolism of AZA, TG and MP. Including the metabolism of AZA to MP using glutathione catalysed by GST(blue arrow), uptake by equilbrative and concentrative nucleotide transporters (ENTs and CNTs) (black arrows), metabolism by various enzymes hypoxanthine-guanine phosphoribosyl transferase (HGPRT), xanthine oxidase (XO), inosine monophosphate dehydrogenase (IMPDH), GMP synthase (GMPS) and thiopurine methyl transferase (TPMT), (blue arrows) and excretion of thio uric acid, (black arrow). Cytotoxic effects via incorporation into DNA, disruption of cell signalling and inhibition of de novo purine synthesis (red arrows), and the site of action of XO inhibitors (*).

TXMP is aminated to TGMP by guanosine monophosphate synthase (GMPS, E.C. 6.3.5.2), although this reaction is less favourable than that of endogenous xanthosine monophosphate (XMP) (Spector, 1975).

TGMP is acted upon by nucleotide kinases to form the other TGNs TGDP and TGTP. TGTP is further processed to form deoxyTGTP which may be incorporated into DNA.

Thiopurine metabolites are methylated by TPMT. These reactions form the methylated metabolites meTIMP, methyl thioguanosine monophosphate (meTGMP) and methyl mercaptopurine (me MP) (Krynetski *et al.*, 1995). There has been extensive research into the activity and genetics of TPMT. A tri-modal distribution of activities has been discovered. Around 88% of the population has a high (normal) TPMT activity, 11% of the population has half or less activity and less than 1% of the population no TPMT activity (Weinshilboum and Sladek, 1980). This has been shown to be a result of a monogenic inheritance of TMPT in a co-dominant fashion. A low TPMT activity has been shown to increase the risk of developing adverse reactions, such as neutropenia, to thiopurines at the standard dose (Coulthard *et al.*, 2002). This is due to patients with low TPMT activity producing more of the cytotoxic TGNs and less of the largely-inactive methylated metabolites. There are around 30 genetic variants which lead to a reduction in, or absence of TPMT activity and increased TGNs from standard thiopurine doses (Appell *et al.*, 2013; Hlavaty *et al.*, 2013). In Caucasians the most common single nucleotide polymorphisms are TPMT*3A and TPMT*3C (Serpe *et al.*, 2009). Structural analysis of some of the mutant alleles has shown that some SNPs produce TPMT proteins which are less stable than wild type protein (Feng *et al.*, 2010). Treatment with MP has also been shown to decrease TPMT expression via a variable number tandem repeat in the TPMT promoter (Alves *et al.*, 2001; Kotur *et al.*, 2012). There have also been cases of patients with extremely high TPMT activity who have either not responded to thiopurine treatment (Ansari *et al.*, 2002) or suffered side effects due to increased methylated metabolite levels (Seinen *et al.*, 2013b). Pre-treatment genetic testing for TPMT is now recommended (Sanderson *et al.*, 2004). If a patient is found to be homozygous or heterozygous for one of the known TPMT reduced-activity alleles (Appell *et al.*, 2013), then either up to a 10 fold reduction of thiopurine dose is recommended or another drug should be

prescribed (Relling *et al.*, 2011). In the case of leukaemia the recommended dose of MP is reduced from 60 or 75 mg/m^2 to 6 or 7.5 mg/m^2 depending on the age of patient and the aggressiveness of their disease. In IBD dosage of thiopurine is often determined by the level of TGNs measured, patients with low TPMT activity tend to receive around half the dose of AZA than those with normal TPMT activity to achieve TGNs over 235 pmol/8 x 10(8) RBCs from 0.9 mg/kg/day to 1.8 mg/kg/day (Gardiner *et al.*, 2008a). Differences in TPMT accounts for about half the variability in response to thiopurine drugs and in some diseases such as in rheumatoid arthritis it has been suggested that it may not be cost effective to determine TPMT activity prior to treatment with thiopurines (Booth *et al.*, 2011, Pavlovic and Zukic, 2010).

MP is also metabolised by xanthine oxidase (XO, E.C. 1.17.1.4). This enzyme converts MP to thioxanthine (TX) and TX to thiouric acid (TUA) which is excreted in urine (Kudo *et al.*, 2010) (Atkinson *et al.*, 1965). XO competes with HGPRT for MP (Kalra *et al.*, 2007). This competition may be clinically significant by reducing the efficacy of MP and AZA in patients with high XO activity (Wong *et al.*, 2007).

Thioinosine triphosphate (TITP) is metabolised by inosine triphosphate phosphatase (ITPA, E.C. 3.6.1.19) to form TIMP (Bakker *et al.*, 2011). ITPA activity ensures that endogenous inosine triphosphate (ITP) is kept to a minimum to reduce the risk of it being inappropriately incorporated into DNA. Low ITPA activity has been shown to increase the ratio of methylated metabolites to TGNs which may lead to decreased efficacy of thiopurines (de Beaumais *et al.*, 2011).

1.1.4 Mechanisms of thiopurine cytotoxicity

It has been demonstrated that there are a variety of causes of thiopurine cytotoxicity; most of these are apparently caused by TGNs. A major cause of cytotoxicity of thiopurines is the conversion of TGNs to deoxyTGNs and incorporation of these into DNA. It has been shown that pharmacologically inhibiting DNA synthesis, and therefore preventing incorporation of TGNs in DNA reduces the efficacy of thiopurines (Lee and Sartorelli, 1981). TGNs incorporated into DNA create structural DNA lesions that may incorrectly base pair with thymine (Bohon and de los Santos, 2003). This is due to the altered hydrogen bonding properties of the thiol group compared to the endogenous

oxygen (Figure 1.1). This allows only two hydrogen bonds to form instead of the usual three formed by endogenous guanine allowing pairing with thymidine. These lesions trigger mismatch repair (MMR) systems which begin futile repair attempts and result in apoptosis being triggered (Swann *et al.*, 1996, Karran, 2006). MMR-deficient cells are also susceptible to killing by thiopurine drugs. The involvement of homologous recombination (HR) in the toxic effects of thiopurine drugs has been shown. Rajesh *et al.* (2011) showed that cells with deletion of Rad 51d, needed for HR, are hypersensitive to TG; this is rescued by co-deletion of Mlh1, part of the MMR pathway. However these cells show increased chromosomal instability, suggesting that thiopurines are still incorporated into DNA. Thiopurine drugs can selectively kill BRCA-2-defective tumours, BRCA-2 protein is part of the HR pathway (Issaeva *et al.*, 2010).

The incorporation of methyl TGNs into DNA has been demonstrated to block transcription, which may also contribute to cytotoxicity of thiopurines (Changjun *et al.*, 2012). Incorporation of TGNs and methylTGNs into RNA may reduce the rate and accuracy of transcription, and may reduce cell viability (You *et al.*, 2012).

TGNs do not act only via their incorporation into nucleic acids but also interfere with G-protein signalling. Studies have shown that the interaction of TGTP with the Rac/Vav signalling pathway, in place of endogenous GTP is cytotoxic (Tiede *et al.*, 2003). CD28 activation of T-cells usually results in pro-survival signals via the GTP transfer between Vav and Rac, activating Rac which then acts via the MEKK pathway. However if TGTP binds to Rac it does not activate Rac and so apoptotic signals prevail leading to cell death (Heo and Hong, 2010).

As well as the various cytotoxic effects of TGNs, methylated thiopurine metabolites have cytotoxic effects. meTIMP inhibits *de novo purine synthesis* (DNPS) by inhibiting phosphoribosylpyrophosphate amidotransferase (E.C. 2.4.2.14) (Tay *et al.*, 1969; Benson *et al.*, 1970; Mario *et al.*, 1993). This reduces the availability of endogenous purines, which are essential to cell growth and survival. Also the reduction in endogenous purines may amplify the influence of the thiopurines by increasing the substitution of thiopurines for endogenous purines in processes such as DNA production. This process contributes to the sensitivity of cells to MP but not TG (Dervieux *et al.*, 2001).

Methyl thio-adenosine phosphorylase (MTAP, E.C. 2.4.2.28) is an enzyme of methionine and adenosine salvage. MTAP-deficient cells are more sensitive to thiopurine drugs (Coulthard *et al.*, 2011). Its deletion increases cellular reliance on DNPS probably due to a build-up of methyl thio-adenosine which is a feedback inhibitor of adenine, methionine and spermine recycling pathways (Basu *et al.*, 2011). DNPS is inhibited by methylated thiopurine metabolites. Also increased MTAP activity decreases TPMT activity and methylated metabolites; in spite of this MTAP negative cells are more sensitive to MP than MTAP positive cells (Coulthard *et al.*, 2011). Finally, thiopurine treatment has been shown to alter DNA methylation *in vitro*. This may cause cytotoxicity, but this does not appear to depend on TPMT activity (Hogarth *et al.*, 2008; Wang and Wang, 2010a; Yuan *et al.*, 2011).

1.1.5 Current Clinical use

MP and occasionally TG are used in the treatment of paediatric T-lineage acute lymphocytic leukaemia (T-ALL). The incidence of ALL is 1.36 per 100 000 in the United States of America for 1975-2011 (Surveillance). The intermediate recommendations for treatment of T-ALL in children under 10 without high risk factor between UKALL 2003 and UKALL 2011 trials suggested the use of 75 mg/m^2 from week 5 of induction treatment in combination with dexamethasone, vincristine, pegylated asparginase and methotrexate, the same dose of MP is used during interim maintenance phase in combination with the other drugs mentioned above and danorubicin, followed by a reduction to 60 mg/m^2 of MP in combination with other drugs during delayed intensification, MP is increased back to 75 mg/m^2 for 2 or three years of maintenance therapy, again in combination with other drugs. It is recommended that patients homozygous for TPMT low activity alleles are given 10% of the normal dose, 6 or 7.5 mg/m^2 (Clinical Trial Service Unit) or 10% of the normal dose, 7.5 mg/ m^2, on alternate days instead of daily (Lennard *et al.*, 1997). The same doses of MP are recommended for higher risk patients but with different combinations of other drugs (Clinical Trial Service Unit). It has also been suggested that TG may replace MP for patients lacking TPMT, as TG metabolism by TPMT is less critical to TG. However there is an increased risk of veno-occlusive disease when using TG (Sati *et al.*, 1982). Higher thiopurine doses have been associated with improved outcome but also with secondary malignant neoplasm

(Schmiegelow *et al.*, 2009). Given that too little thiopurine or too much have been shown to have adverse outcomes it is important that the dose of these drugs is optimised for individual patients.

AZA, and more occasionally TG and MP are used in the treatment of inflammatory conditions such as IBD. IBD is a group of conditions; ulcerative colitis, inflammation of the colon, and Crohn's disease, inflammation of the small intestine. Both are due to an inappropriate activation of the mucosal immune system damaging the gut. The incidence of Crohn's disease in Europe varies between countries from 0.87 to 9.8 cases per 100 000 people (Loftus, 2004). Ulcerative colitis has a slightly higher incidence between 1.5 and 20.3 cases per 100 000 (Loftus, 2004). IBD is treated with a combination of drugs and surgery. Drugs used include infliximab, an anti-tumour necrosis factor (TNF) alpha monoclonal antibody, corticosteroids and immunomodulators including methotrexate and the thiopurines, in particular AZA (Bressler *et al.*, 2015). Thiopurines are often used as steroid-sparing agents, this reduces dose of corticosteroid used and therefore the harmful side effects such as weight gain and growth stunting are minimised. Newer drugs such as Infliximab are mono-clonal antibodies targeting TNF to reduce inflammation. Although these drugs become effective more rapidly than thiopurines they must be administered either intra-venously or sub-cutaneously, not orally, making them less convenient and some studies have associated them with an increased risk of infection.

There have been several strategies developed to improve the response of IBD patients to thiopurines, reducing or removing the necessity for them to be treated with other drugs or surgery. One strategy is to combine AZA with allopurinol, this is discussed in detail later. Another strategy is to administer the same daily dose of AZA, MP or TG split into two doses to be taken morning and evening. Splitting the dose of AZA or MP into two daily doses rather than one has been shown to maintain therapeutic TGN levels whilst reducing levels of methylated metabolites (Shih *et al.*, 2012). It is as yet unclear why there is this effect on the relative amounts of different metabolites of thiopurine metabolites.

1.1.6 Optimisation of thiopurine therapy using allopurinol

Both TGNs and methylated thiopurine metabolites contribute to the cytotoxicity of thiopurines and their side effects. However whilst TGN levels correlate most

strongly with efficacy, methylated metabolite levels correlate mostly with toxic side effects, particularly hepatotoxicity (Dubinsky *et al.*, 2002; Derijks *et al.*, 2004; Gardiner *et al.*, 2008b). In some patients who do not respond to AZA or MP, dose escalation results in the same TGN levels but higher methylated metabolite levels and side effects, these patients are referred to as hyper-methylators or shunters (Gardiner *et al.*, 2008b) (Ansari *et al.*, 2002; Dubinsky *et al.*, 2002). It is important to note that although a correlation between hypermethylation and hepatoxicity has been shown a mechanistic explanation has not yet been found. It has been suggested that hyper-methylation is not necessarily caused by higher than usual TPMT activity (Seinen *et al.*, 2013b). Recently a reduced dose of AZA or occasionally MP combined with low dose allopurinol has been shown to reduce the level of methylated metabolites whilst maintaining or increasing TGNs in erythrocytes (RBCs) to desirable levels, from levels of methylated metabolites over 5,700 pmoles/8 x 10^8 RBCs and under 235 pmoles/ 8 x 10^8 RBCs of TGNs (Sparrow *et al.*, 2005; Leung *et al.*, 2009; Schmiegelow *et al.*, 2010; Brackett *et al.*, 2014).

Allopurinol is a HX analogue that, along with its longer lasting metabolite oxypurinol acts as an XO inhibitor (Elion, 1966; Elion, 1989), (Hande *et al.*, 1978). It is used to reduce uric acid (UA) concentrations in gout (Ts'ai-Fan and Gutman, 1964) (Elion, 1989). It was initially developed with the assumption that by blocking the excretion of thiopurines their efficacy would be increased (Elion *et al.*, 1988). Initial trials of the combination of allopurinol with thiopurines showed increased toxicity in humans rather than the hoped for increase in therapeutic effects. This led to allopurinol being used only in the treatment of gout and high urate for several decades (Coffey *et al.*, 1972; Walker *et al.*, 1973; Tterlikkis *et al.*, 1983) (Tterlikkis *et al.*, 1983; Elion *et al.*, 1988) and it was recommended that if allopurinol treatment for high urate levels was needed in combination with immunosuppression thiopurines should be avoided or used at a reduced level. This approach is still recommended by some clinicians (Scheffert and Raza, 2014). It was not until the 2000’s that a low dose AZA and allopurinol regime has been recommended in the treatment of IBD (Sparrow *et al.*, 2005), this followed use of this drug combination in renal transplant (Chocair *et al.*, 1993). This regime is normally recommended for patients with a high methylated metabolites and low TGNs which do not respond to dose increases.

The dose of AZA is reduced to between half and one quarter of its previous dose, for example in the initial Sparrow study in 2005 the average AZA dose dropped from 188 mg/day to 88 mg/day; this was combined with 100 mg of allopurinol, instead of the standard gout treatment dose of 300 mg. This has been shown to increase TGNs to above 235 pmoles/8 x 10^8 RBCs, a therapeutic level, and reduce the amount of methylated metabolites. This alteration in metabolite levels was shown to be effective at decreasing the need for steroids with minimal side effects such as leukopenia (Seinen *et al.*, 2013a).

The effect on the ratio of methylated metabolites/TGNs is unexpected as the inhibition of XO, the alternative metabolic pathway for MP, would be expected to increase the concentration of all MP metabolites other than TX and TUA.

It has been suggested that allopurinol increases the concentration of TX and that this inhibits TPMT (Blakera *et al.*, 2012), decreasing the relative concentration of methylated metabolites produced by TPMT. Allopurinol itself does not inhibit TPMT (Seinen *et al.*, 2013a). Chronic treatment with allopurinol and AZA produces an increased HGPRT activity in RBCs (Seinen *et al.*, 2011). XO also has aldehyde oxidase effects and allopurinol also inhibits aldehyde oxidase (Krinetsky *et al.*, 1972), a closely related enzyme and affect the reactive oxygen species (ROS) production by both enzymes. It has been suggested that the reduced adverse events observed in patients on the combination of AZA and allopurinol may be due to a reduction in cellular damage by ROS and it has been shown that allopurinol reduces AZA associated ROS in rat hepatocytes (Al Maruf *et al.*, 2014). It may be that ROS are particularly important to the cytotoxic effects of thiopurines on DNA via oxidation of incorporated TGNs (Brem and Karran, 2012) and that by reducing the amount of ROS excess DNA damage is prevented.

Clearly it is important to understand the mechanistic basis of interaction between thiopurine metabolism and allopurinol in order to improve the clinical use of this drug combination.

1.1.7 Measurement of thiopurine metabolites

The metabolism of thiopurines (Figure 1.2) relies on several enzymes which may vary in activity between individuals. This variation has a clinical relevance. Measurements of TGNs and meTIMP are normally made in RBCs, an abundant

cell type but not the cell type assumed to be the target of thiopurine drugs; RBCs also lack the necessary enzymes for production of TGNs and, therefore, must accumulate TGNs released from other cell types. Despite the limitations of assaying thiopurine metabolites in RBCs, a correlation has been repeatedly shown between concentration of TGNs and outcome of treatment (Dubinsky *et al.*, 2000; Cuffari *et al.*, 2001; Wright *et al.*, 2004; Ooi *et al.*, 2007). Measurements of methyl mercaptopurine riboside (me MPR), used as representative of methylated inosinic metabolites, indicate the likelihood of hepatotoxicity (Dubinsky *et al.*, 2000; Dubinsky *et al.*, 2002; Derijks *et al.*, 2004; Gardiner *et al.*, 2008b). However, large scale analyses of the utility of thiopurine metabolite measurements do not all find them to be useful (Konidari *et al.*, 2014). Many do but others do not (Osterman *et al.*, 2006; Ooi *et al.*, 2007; Moreau *et al.*, 2014).

There is a method-dependent variation in thiopurine metabolite levels measured using two common methods for TGN measurement (Shipkova *et al.*, 2003), with a 2.6 fold difference between measurements made by the Lennard method (Lennard, 1987), which relies on phenyl mercury adduct formation and sulphuric acid hydrolysis to extract metabolites, and the Dervieux method (Dervieux and Boulieu, 1998c) which uses perchloric acid and dithiothreitol (DTT). Although Shipkova also states that this difference can be reduced to 1.4 fold by using perchloric acid in the Lennard method rather than the previously described sulphuric acid (Shipkova *et al.*, 2003). The different processing strategies used by each method cause a difference in the hydrolysis of TGNs to TG and therefore the reported metabolite levels.

It has been shown that meTIMP and me MPR become 4-amino-5-(methylthio)carbonyl imidazole, which is detected, after preparation by Dervieux's acid hydrolysis method (Dervieux and Boulieu, 1998b; Dervieux *et al.*, 2001). This may make comparison between metabolite levels difficult. Despite these differences in processing, some studies of the correlation between reported metabolite levels and outcome shows that TGN measurement correlates with remission in IBD and leukaemia, indicating that metabolite measurement is a useful clinical tool (Schmiegelow *et al.*, 1995; Osterman *et al.*, 2006; Ooi *et al.*, 2007). A more direct measurement of thiopurine

metabolites in more-clinically-relevant cell types may prove a more sensitive indicator of clinical efficacy.

1.1.8 Systems biology

Systems biology is an increasingly popular approach to studying biology. Traditional biochemical and genetics approaches have focussed on detailed studies of individual components of biological systems, for example a protein. In contrast systems biology as described by Hood (Ideker *et al.*, 2001), seeks a more integrated picture of an organism as a whole, “it investigates the behaviour and relationships of all of the elements in a particular biological system while it is functioning.” (Ideker *et al.*, 2001). It is a way of understanding and utilising the large quantities of complex data, such as genome wide association studies and proteomics studies, becoming available to biologists. Two complementary approaches are taken by systems biologists. The top-down approach uses large datasets to investigate the interactions of components of biological systems often without initial hypotheses. The bottom-up approach explores current hypotheses about a system. It integrates previous data, often from several sources along with new data to validate or refute old ideas and develop new ones about a biological system. Systems biology uses theories and techniques, such as systems control theory, from engineering to carry out these approaches.

A bottom up approach, computational dynamic modelling allows biological systems to be explored with *in silico* experiments, “providing predictions to be tested by *in vivo* and *in vitro* studies. to predict the dynamics of systems so that the validity of the underlying assumptions can be tested” (Kitano, 2002a). Mathematical modelling of biological systems, reducing them to a set of mathematical equations that approximates their behaviour to aid description and understanding, has been used for many decades. It has been used in enzyme biochemistry and pharmacology, for example Michaelis-Menten kinetics (Michaelis and Menten, 1913) approximated enzyme substrate binding with their equation enabling the study of reaction kinetics. However with the invention of computers in the 1950’s larger, more complex sets of equations could be solved and analysed. For example Turing’s description of morphogen activity (Turing, 1952) during development. The increasing power of computers over the last few decades is a key factor that has led to an explosion in the

number of computational models of biological systems. The BioModels database website listed 530 curated and 655 non-curated models in September 2014 that have been manually created plus over 100 000 created automatically from online databases (*http://www.ebi.ac.uk/biomodels-main/#*; Chen *et al.*, 2010). These models have contributed to a fuller understanding of the emergent properties of networks and their motifs. For example feedback loops give rise to certain behaviours such as signal amplification, switching or oscillations as described by (Alon, 2007).

Model building is often part of a cycle of modelling and experimentation in which *in vitro*, *in vivo* and *in silico* stages validate and inform each other. Modelling suggests future experiments and experiments provide data for model validation and expansion.

Dynamic computational models have been successfully used describe endogenous purine metabolism (Curto *et al.*, 1997; Curto *et al.*, 1998a; Curto *et al.*, 1998b) and the effects of pharmacological inhibition of IMPDH on concentrations of endogenous purines (Thomas, 2010). Curto *et al*. used their model to explore possible therapeutic options for enzyme deficiencies of purine metabolism (Curto *et al.*, 1998a).

A previously published study of the cytotoxic effects of TGN incorporation used dynamic modelling of the cell cycle and large scale gene expression analysis to examine the mechanisms of loss of sensitivity of cell culture cell lines to MP (Panetta *et al.*, 2006). The process of cell cycle arrest and apoptosis was modelled and data from sensitive and insensitive cells was used to estimate the kinetic constants of the model. Comparison of these constants enabled differences in response to TGN incorporation to be analysed showing that differences in the progress through the cell cycle were important. This result was confirmed by gene expression differences in the sensitive and resistant cells in proteins involved in the regulation of the cell cycle.

The pharmacokinetics and metabolism of thiopurines in patients have also been analysed using computational modelling (Fransson *et al.*, 2006; Fransson *et al.*, 2007). However the study was inconclusive, the model had a limited ability to fit data from patients about the changing concentrations in RBCs of meTIMP and TGNs in patients newly treated with AZA. The relatively small number of

metabolites analysed compared to the complexity of thiopurine metabolism along with the complications of metabolism in different cells and the exchange of metabolites between cell types and extracellular compartments meant that this model was unsuccessful.

Systems biology is also associated with a move towards personalising medicine. This includes better understanding the implications of genomic, and other large scale data about patients and also by modelling the course of diseases and the effects of pharmacological interventions. This is becoming known as “4P medicine” standing for predictive, personalised, preventative and participatory medicine, and aims to improve healthcare for individuals and eventually to reduce costs by making medicine more proactive (Hood and Friend, 2011). An example of this is a computational model of apoptosis built using data from cell lines (Huber *et al.*, 2007) that has been applied to samples from patients with colon cancer. It was shown to predict their response to treatment (Hector *et al.*, 2012). This may, soon, be used clinically to direct treatment.

The metabolism of thiopurines and their downstream effects are complex and, despite long term *in vivo* and *in vitro* study, are still not fully understood. This lack of understanding means that not all patients can benefit from treatment with thiopurine drugs. The purpose of this project was to complement new and existing *in vitro* studies with *in silico* studies using systems biology techniques. It was envisaged that by integrating previous knowledge of the mechanisms of thiopurine metabolism into a computational model the interactions between different parts of thiopurine metabolism and their relative influence on the production of thiopurine metabolites would be better understood. This model could be used to predict the effects of altering thiopurine metabolism on the production of cytotoxic metabolites, for example exploring the effects of differing enzyme activities and the interactions of thiopurines with other drugs. Increasing understanding of thiopurine metabolism could eventually lead to improved treatment of patients by taking into account their ability to metabolise thiopurine drugs. This personalisation of drug dosage would improve the quality of life of patients, and reduce the cost of adverse drug reactions to the healthcare system.

1.1.9 Outlook

The complexity discussed in the section on thiopurine metabolism particularly, the unexpected and unexplained effects of the combination of allopurinol with AZA or MP indicates the need for a computational modelling approach to understanding thiopurine metabolism. The previous successful use of computational modelling to understand the effects of thiopurines on cell survival (Panetta *et al.*, 2006) and the analysis of purine metabolism (Curto *et al.*, 1997; Curto *et al.*, 1998a; Curto *et al.*, 1998b; Thomas, 2010) provided confidence that informing dynamic modelling with *in vitro* data would be likely to be a successful tool for examining thiopurine metabolism. However a previous study attempting to model thiopurine metabolism in patients showed the limits of a computational modelling approach (Fransson *et al.*, 2006; Fransson *et al.*, 2007). This model was unable to replicate data about whole body thiopurine metabolism. This was largely due to the fact that data was limited to one cell type and only two thiopurine metabolites. Given that the model was attempting to replicate the effects of reactions that include many other metabolites and their transport between many cell types, these data were inadequate. A dynamic model of thiopurine metabolism in cultured cells would be more likely to provide a tractable problem as it has a more limited set of transport reactions and is more amenable to the generation of experimental data on metabolite levels for validating the model. It is also the case that MP may provide a useful model for thiopurine metabolism in general. The metabolism of MP is the same as that of AZA except for the production of MP from AZA. As the activation of AZA is largely hepatic, intestinal and extra-cellular it would be difficult to study in a cell culture system of only one cell type.TG is less commonly used than MP and therefore is of less clinical relevance, also the metabolism of TG lacks several of the steps of MP metabolism allowing less scope for study. For this reason MP has been studied most extensively during this project.

1.1.10 Aims

1. To build a dynamic computational model of *in vitro* thiopurine metabolism, using MP as an example
2. To generate data on MP metabolites in cultured cells to facilitate model building and validation
3. To assess the cellular effects of allopurinol, alone and with MP *in vitro*

4. To use the model to assess the possible mechanisms of interaction of MP metabolism and allopurinol

Chapter 2. Cytotoxicity of thiopurines and XO inhibitors

2.1 Introduction

The aim of this project was to produce a computational model of thiopurine metabolism to predict the production of different toxic metabolites and to elucidate the relationship between metabolites and mechanisms of cell death. Prior to producing data in cultured cells to inform the model it was necessary to determine that the cell line used was sensitive to MP, the drug whose metabolism would be modelled, and also to determine if allopurinol, a XO inhibitor relevant to thiopurine metabolism, caused cytotoxicity alone or in combination with MP. To this end cell viability was measured, after treatment with the drugs, by the methyl tetrazolium sulphate (MTS) assay (Barltrop and Owen, 1991).

2.1.2 Utility of cell culture studies in the investigation of thiopurine drugs

Many studies have utilised *in vitro* cell culture to elucidate the effects and the metabolism of the thiopurine drugs including effects of various enzymes MTAP (Coulthard *et al.*, 2011), IMPDH (Yamada *et al.*, 1990; Vethe *et al.*, 2008) and in particular the clinically-relevant enzyme TPMT (Dervieux *et al.*, 2001; Coulthard *et al.*, 2002; Misdaq *et al.*, 2012) on the sensitivity to AZA, MP, TG and their metabolites (Mario *et al.*, 1993; Kalra *et al.*, 2007). Studies of the downstream effects of various metabolites using cultured cells have suggested possible mechanisms by which the thiopurine metabolites cause cytotoxicity (Krynetski *et al.*, 2001; Hogarth *et al.*, 2008; Heo and Hong, 2010; Rajesh *et al.*, 2011) and by which cells may develop resistance (Fotoohi *et al.*, 2006b; Panetta *et al.*, 2006). One of these studies used a combination of *in vitro* studies and *in silico* modelling to enhance the understanding of the development of thiopurine resistance (Panetta *et al.*, 2006). The computational model in this study suggested that differences in the process by which cells enter apoptosis may be responsible for differences in sensitivity to MP, this was confirmed by differences in expression of genes involved in apoptosis between sensitive and resistant cells.

Cell culture experiments allow better controlled experiments using specific cell types than *in vivo* studies, as factors such as genetic background, and drug delivery time cannot be controlled with *in vivo* studies. However, *in vitro* studies allow only limited conclusions to be drawn about the metabolism of drugs in

patients as other factors such as hormones, blood circulation and metabolism by multiple cell types may be important.

2.1.3 Thiopurine drug metabolism by T-ALL derived cell lines

For these experiments MOLT-4 and Jurkat cell lines were used; these are both lymphocyte-derived cell lines from patients with T-ALL. T- ALL cells are considered to have the ability to metabolise thiopurine drugs fully (Figure 1.2). Thiopurine drugs are metabolised by XO. MP is metabolised by XO, first to TX then to TUA. This metabolism has been confirmed to occur in Jurkat cell lysates (Kalra *et al.*, 2007). It was therefore assumed in this project that MOLT-4 cells, a cell line of similar origin, would also show this part of the metabolism. MOLT-4 cells have IMPDH activity (Yamada *et al.*, 1990; Vethe *et al.*, 2008) and can therefore metabolise TIMP to TXMP. The action of HGPRT, IMPDH, GMPS and XO in MOLT-4 cells is further supported by the fact that, after incubation with MP, the metabolites TIMP, TXMP, TGMP and TUA or TX are all detectable in MOLT-4 cells (Zimm *et al.*, 1985). It has also been shown that MOLT-4 cells are able to incorporate TGN into DNA after incubation with MP and that they also produce meTIMP showing TPMT activity (Bökkerink *et al.*, 1993). The metabolism of thiopurine drug metabolites by TPMT has been shown to occur in CCRF-CEM and Jurkat cells lines (Dervieux *et al.*, 2001), both of which are T-ALL cell lines suggesting that T-ALL cell lines can be used to assess this aspect of thiopurine metabolism.

The aim of therapy with thiopurine drugs is to reduce the number of lymphocytes in autoimmune diseases or the number of leukemic blast cells in T-ALL. As MOLT-4 and Jurkat cells metabolise thiopurine drugs to their cytotoxic metabolites and are from a clinically-relevant cell type, these appear to be suitable models to use to generate data for a model of thiopurine metabolism. However MOLT-4 cells are tetraploid therefore their incorporation of TGNs into DNA, and its cytotoxic effects, may differ from normal diploid cells due to the difference in total DNA content. This difference must be borne in mind when drawing conclusions about the effects of thiopurines on cells resulting from the incorporation of TGNs into DNA from studies using MOLT-4 cells.

2.1.4 XO inhibitors

The aim of this project was to investigate the interaction between allopurinol and thiopurines experimentally in cultured cells and using a computational

model. The objective of these studies was to explore mechanisms by which allopurinol may modulate the effect of thiopurines in patients. Therefore, it was necessary to assess the cytotoxicity of allopurinol alone and in combination with MP.

Although it is more common to combine AZA with allopurinol clinically, MP is an activated metabolite of AZA and offered a simpler system to study the effects of allopurinol on thiopurine metabolites. Furthermore, the clinical effect of MP with allopurinol was shown to be similar to that seen with AZA (Brackett *et al.*, 2014).

In addition to allopurinol the cytotoxicity of febuxostat and oxypurinol was assessed. These are alternative XO inhibitors; oxypurinol is a metabolite of allopurinol but febuxostat is not a purine analogue. It may be useful to compare the actions of these different types of XO inhibitor to assess which effects stem from the fact that allopurinol mimics HX and which effects are purely due to XO inhibition.

2.1.5 Aims

1. To confirm the sensitivity to thiopurine drugs of MP and AZA in MOLT-4 and Jurkat cell lines using the MTS assay.
2. To assess the possible cytotoxic effects of allopurinol, its active metabolite oxypurinol and febuxostat in the same cell lines alone and in combination with MP, using the MTS assay.

2.2 Methods

2.2.1 Cell lines

Three cell lines were used throughout this project; all are T-ALL derived cell lines. Two were derived from Jurkat cells, a pseudo-diploid cell line expressing the human T cell antigen from the peripheral blood of a 14-year old male patient. Jurkat-MTAP$^{-/-}$(Clontech™), expresses wild-type TPMT, and is MMR negative and MTAP deficient. Jurkat-MTAP+/+ are Jurkat-MTAP-/- cells stably transfected with MTAP cDNA under the control of a tetracycline promoter, previously engineered by Dr. Sally Coulthard. The third cell line was MOLT-4, derived from a 19 year old T-ALL patient after drug treatment and relapse. MOLT-4 cells are hyper-tetraploid, express MTAP and wild type TPMT but lack expression of p53. As previously discussed tetraploidy may affect the response of MOLT-4 cells to thiopurines due to altered DNA content.

Jurkat-derived cell lines were grown in Roswell Park Memorial Institute (RPMI) medium containing 10% dialysed foetal calf serum (Cambrex™) and 500µg/ml Geneticin® (GIBCO-BRI but with the addition of 1 mg/ml Hygromycin B (Invitrogen™) for Jurkat-MTAP+/+ cells. Expression of MTAP was induced by the addition of 2 mg/ml Doxycycline to Jurkat-MTAP$^{+/+}$ and as a negative control the same drug was added to the Jurkat-MTAP-/- cells. MOLT-4 cells were grown in RPMI with 10% normal foetal calf serum (Cambrex™) and no other drugs. All cells were maintained in an incubator at 37˚C and 5% CO_2. All cell lines have a doubling time of approximately 24 hours. Cells were maintained at a density of between $1x10^5$ and $2x10^6$/mL.

2.2.2 MTS cell growth assay

To measure the proportion of viable cells in drug sensitivity assays, a 96-well plate format MTS assay, CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Southampton, United Kingdom), was used. This is a colorimetric assay which relies on the ability of NAD(P)H oxidoreductases in viable cells to reduce the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium to a soluble formazan product detectable at 490-560nm (Barltrop and Owen, 1991). To perform this assay, 25 µL of MTS reagent was added to wells of a 96 well plate containing 200 µL of either blank cell culture medium or cultured suspension cells in medium. Plates were wrapped to protect from light and incubated at 37 ˚C for 2

hours. The colour change was detected using a FLUOstar Omega Microplate reader (BMG LabTech, Oretenberg Germany) set to 490 nm absorbance detection mode.

2.2.3 Drug preparation

MP, allopurinol and oxypurinol were initially suspended in 0.1 M NaOH at concentrations of 50 mM. AZA was initially suspended in DMSO at 100 mM febuxostat was dissolved in DMSO at 50 mM. All drugs and reagents were purchased from Sigma (Gillingham, UK) except febuxostat was purchased from Carbosynth (Compton, Berkshire, UK) and all stock solutions were stored at -80 ^{0} C. Stock solutions of each drug were diluted in relevant medium at concentrations shown in Table 2.1. A control vehicle (CV) solution was also prepared by diluting NaOH or dimethyl sulfoxide (DMSO) in relevant medium to give the same concentration of vehicle as the concentration of vehicle used in the medium containing the drug. A serial dilution of 1:2 of medium containing the drug and CV was made, producing a range of eight concentrations of drug for sensitivity testing.

Drug	Final Concentration (concentration in medium)	Dilution in Medium Before Addition to Cells
AZA	100 μM (200 μM)	1:500
MP	50 μM (100 μM) 100 μM (400 μM)	1:500, 1:125
Allopurinol	1 mM (2 mM and 4 mM for dilution with 6-MP) 100 μM (200 μM and 400 μM) 10μM (20 μM and 40 μM)	1:25 and 1:12.5 1:250 and 1:125 1:2500 and 1:1250
Oxypurinol	1 mM (2 mM)	1:25
Febuxostat	1 mM (2 mM)	1:25

Table 2.1 Dilutions of drugs added to cell plates.

2.2.4 Drug Sensitivity Assay

Cells were plated in rows B-G of columns 3-11 of a 96 well microplate, 100 µL of 5 x 10^5 cells/mL were used. Rows B-G of column 2 was filled with 200 µL of medium. All other wells were filled with sterile phosphate buffered saline (PBS), pH 7.45, 10 mM phosphate (Gibco life Technologies, Paisley, UK). Plates were incubated for 24 hours at 37 ^{0}C. 100 µL of CV or drug was then added to each cell-filled well and each of the eight serially diluted drug concentrations was added to one column of wells (6 wells). In the case of combination studies with allopurinol one column of cells had allopurinol at 2 mM, 200 µM or 20 µM, and dilution 1:250 0.1M NaOH, to give final concentrations of 1 mM 100 µM and 10 µM of allopurinol, each in two wells. Serial dilution of MP from a top concentration of 100 µM (concentration when added to cells) was added to allopurinol at a concentration (on cells) of 1 mM, 100 µM or 10 µM, each concentration such that each combination of drugs was in two wells per plate. Plates were then incubated for 72 hours, approximately equivalent to 3 doublings, before being assayed by MTS assay.

2.2.5 Statistical analysis

Data were processed using Microsoft Excel and R (Team, 2014). Means of percentage survival, compared to the survival of cells grown with only CV, means of six wells were determined in Microsoft Excel.

The data for cell survival in response to XO inhibitors were initially fitted to either a linear (1st order) model or a 2nd order polynomial using the lm function in R for response against dose. These two models for each data set were compared by analysis of variance using the anova function in R. If there was no significant difference, at the p-value < 0.05 level, between the two models then the simple linear model was used in further analysis; if there was a significant difference then the 2nd order polynomial was used. A new model (either linear or 2nd order polynomial, as appropriate) was then created for each drug in each data set including the biological repeats as a variable. The two models were then compared using the anova function as above. If there was no significant difference, at the p-value < 0.05 level (indicating that the data points within each experiment could be considered as independent in the context of all experiments when considered together), then the anova function was used to derive the significance for the effects of dose using the model of dose versus

response alone for the combined data set. Where models which included the experimental repeats as a factor were significantly different from the simpler model without experiment as a factor, all three biological replicates were used analysed separately using t-tests to assess the significance of individual parameters.

For the sensitivity assay with AZA, MP and MP with allopurinol 4-parameter logistic curves were fitted to each of the 3 biological replicates and effective dose (ED) 50 were determined using the drm function from the drc package in R (Ritz and Streibig, 2005). The mean and standard deviation (SD) of these ED 50 values were determined in R. The difference between AZA and MP was determined using a T-test in R to compare the ED 50 values of each drug. In order to determine if the various combinations of MP and allopurinol differed in their effects from MP alone, analysis of variance using the ANOVA function and a Dunnett's test was used to compare the ED 50 of each of the drug combinations to the ED 50 of MP alone, this accounts for multiple tests being made using one control. This test was performed by modelling the effects of allopurinol dose, as discreet categories not a continuous variable, on the ED 50 values of each drug combination. This model was assessed by analysis of variance using the aov function in R. This model was then subjected to the Dunnett's test using the glht, generalised linear hypothesis test, function from the multcomp package in R described in Hothorn *et al.* (2008).

2.3 Results

2.3.1 Sensitivity of lymphocyte derived cell lines to XO inhibitors

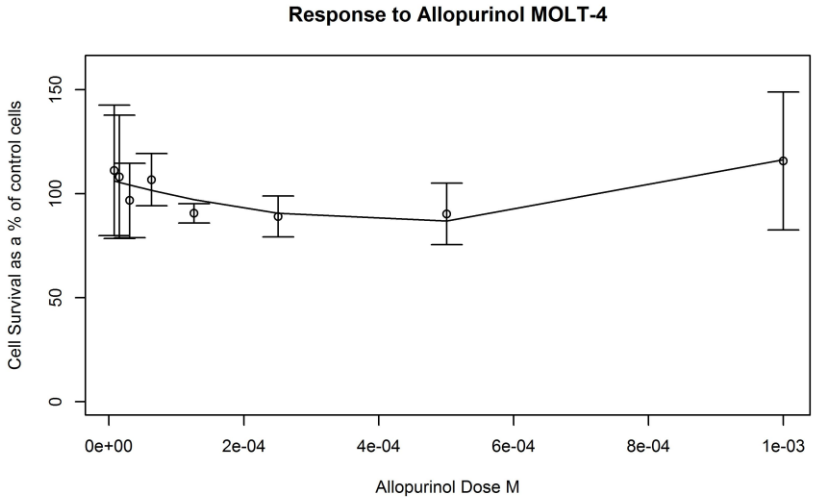
All three cell lines, MOLT-4, Jurkat MTAP -/- and Jurkat MTAP +/+, were tested for their cell survival response to allopurinol (Figure 2.1), its active metabolite oxypurinol (Figure 2.2), and febuxostat (Figure 2.3). This was to inform the setting up of subsequent experiments when growing cells for metabolite measurements to avoid loss of cells due to growth with an XO inhibitor.

The survival of MOLT-4 cells in the presence of allopurinol was best described by a 2nd order polynomial model. Models fitted with and without experimental repeats as a factor were not significantly different, and all experimental data were analysed together as a simple 2nd order model. This model did not show a significant effect of dose (P= 0.1146), indicating that allopurinol had no effect on MOLT-4 cell survival. The response of Jurkat MTAP-/- cells to allopurinol was best fitted by a linear model; however dose significantly increased survival in Jurkat MTAP -/- cells (P=0.0399), although the effect was small and likely to be heavily influenced by the data for the highest dose (Fig 2.1b). The survival of Jurkat MTAP +/+ cells with allopurinol was also best fitted by a simple linear model with no effect of experimental repeat, and there was no significant effect of allopurinol dose on the survival of Jurkat MTAP +/+ cells (P= 0.762).

The survival of MOLT-4 cells cultured with oxypurinol was best fitted by a linear model in which experimental repeat had to be included as a factor. The effect of dose was not significant in two out of the three biological repeats, one repeat showed a significant increase in cell survival (P= 0.00239). Therefore, oxypurinol had a somewhat variable effect on MOLT-4 cell survival but did not significantly reduce cell survival. The response of Jurkat MTAP-/- cells to oxypurinol was fitted by a linear model with experimental repeats as a factor, but since only one repeat showed a significant reduction in cell survival (P =0.000798) the data suggest that oxypurinol does not, overall, have a significant effect on cell survival. Conversely, the effect of oxypurinol on Jurkat MTAP +/+ cells was linear with no effect of repeat, with a small but significant decrease in viability with increased dose (P= 0.000118) (Figure 2.2c).

The effects of febuxostat on MOLT-4 and Jurkat MTAP -/- cells were best described by linear models with experimental repeat as a factor. Febuxostat

caused a significantly dose dependent reduction in MOLT-4 and Jurkat MTAP -/- cell survival ($P<0.05$) (Figure 2.3b) Jurkat MTAP +/+ cells were also affected by febuxostat; in this case a 2nd order polynomial with with no experimental repeat as a factor was the best fitting model in which febuxostat dose significantly decreased cell viability ($P<0.001$).



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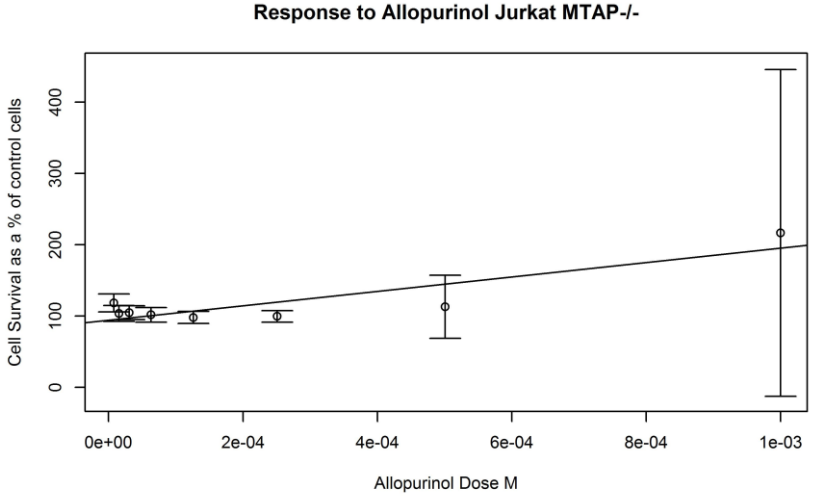

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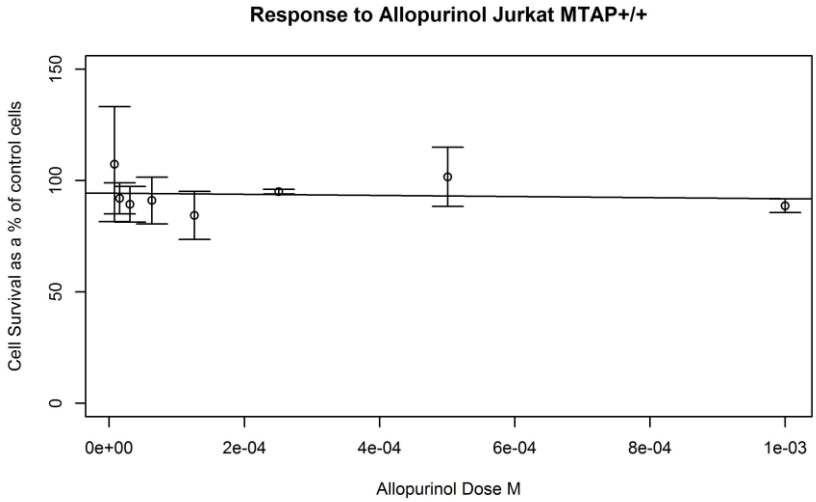
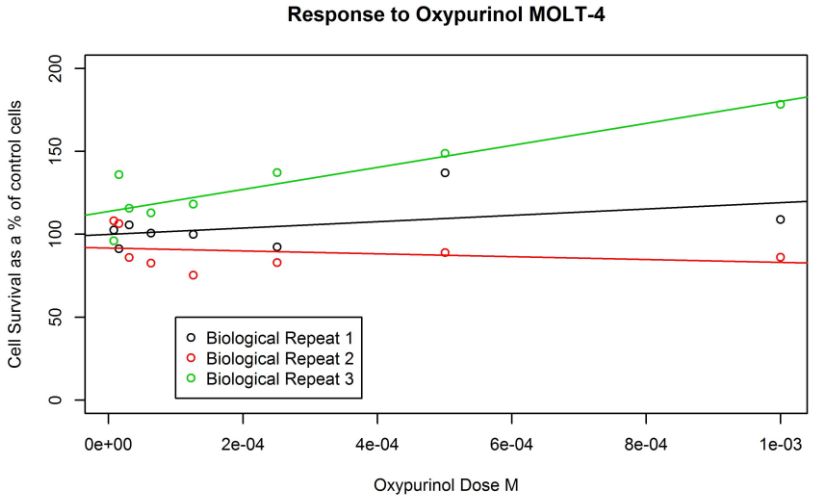


c

Figure 2.1 Cell survival curves showing sensitivity to allopurinol points are means, error bars 1 SD and curves are linear or 2nd order polynomial models fitted in R **a** MOLT-4 2nd order polynomial (n=3) **b** Jurkat MTAP-/- linear model (n=3) **c** Jurkat MTAP +/+ linear model (n=3)



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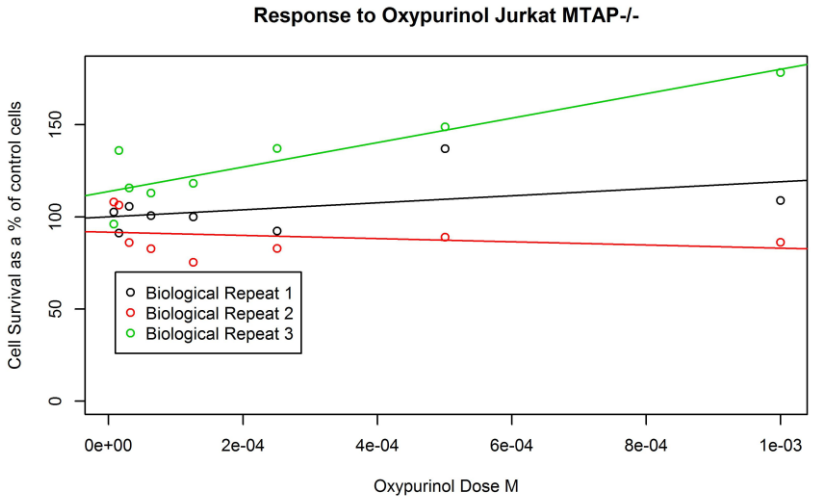

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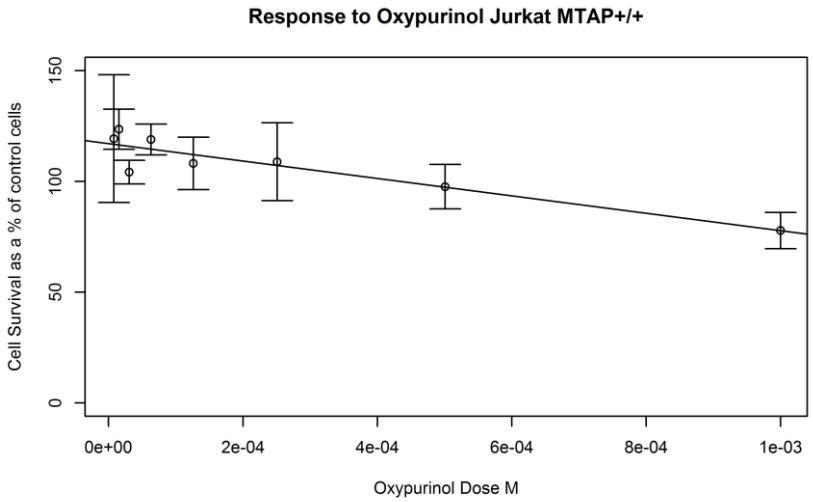
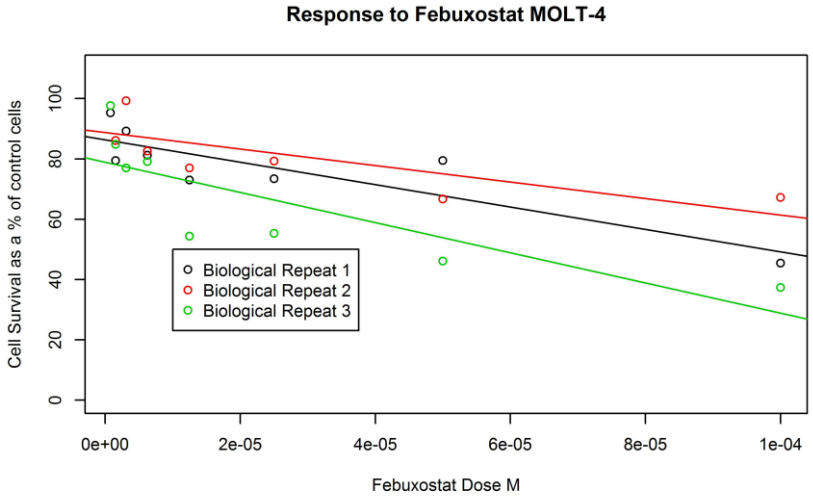


c

Figure 2.2 Cell survival curves showing sensitivity to oxypurinol curves are linear models fitted in R **a** MOLT-4 points biological replicates, lines models fitted to each biological replicate **b** Jurkat MTAP-/- points biological replicates, lines models fitted to each biological replicate **c** Jurkat MTAP +/+ points are means, error bars 1 SD



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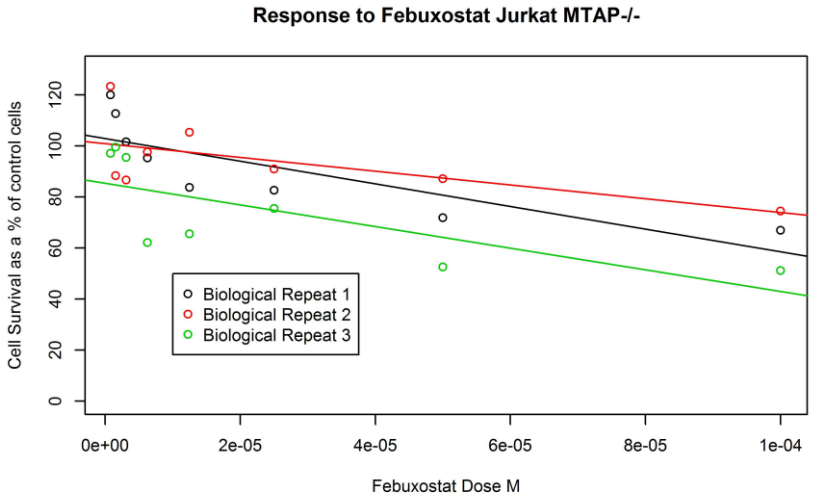

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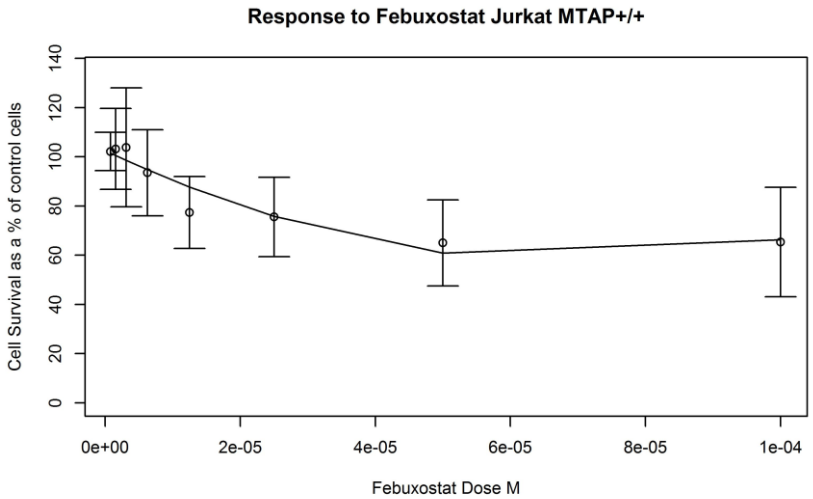


c

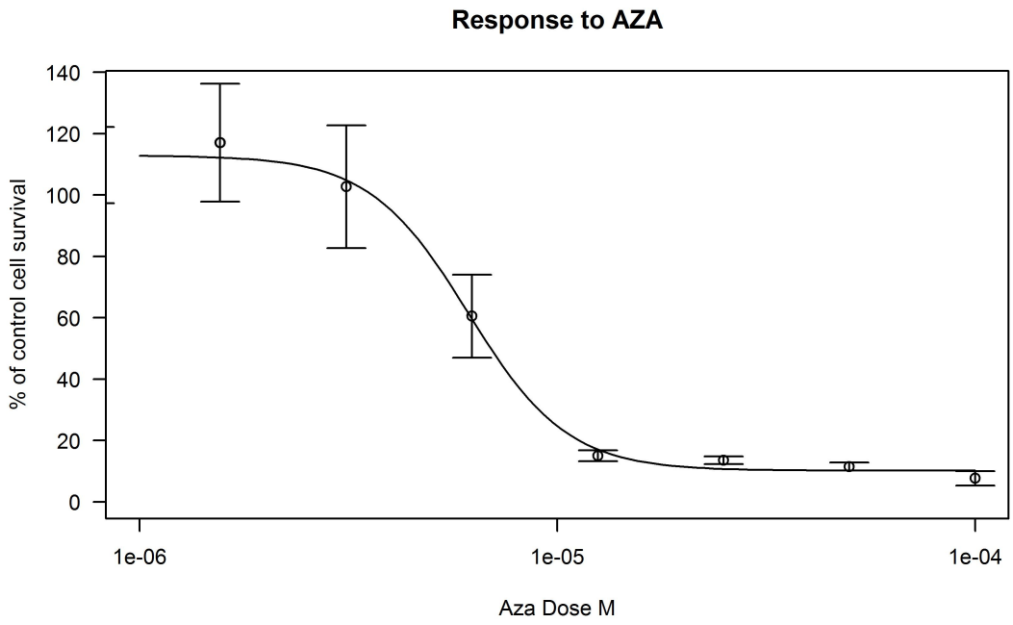
Figure 2.3 Cell survival curves showing sensitivity to febuxostat curves are linear or 2nd order polynomial models fitted in R **a** MOLT-4 points biological replicates, lines models fitted to each biological replicate **b** Jurkat MTAP-/- points biological replicates, lines models fitted to each biological replicate **c** Jurkat MTAP +/+ 2nd order polynomial (n=3) points are means, error bars 1 SD

2.3.2 Sensitivity of MOLT-4 cells to thiopurine drugs

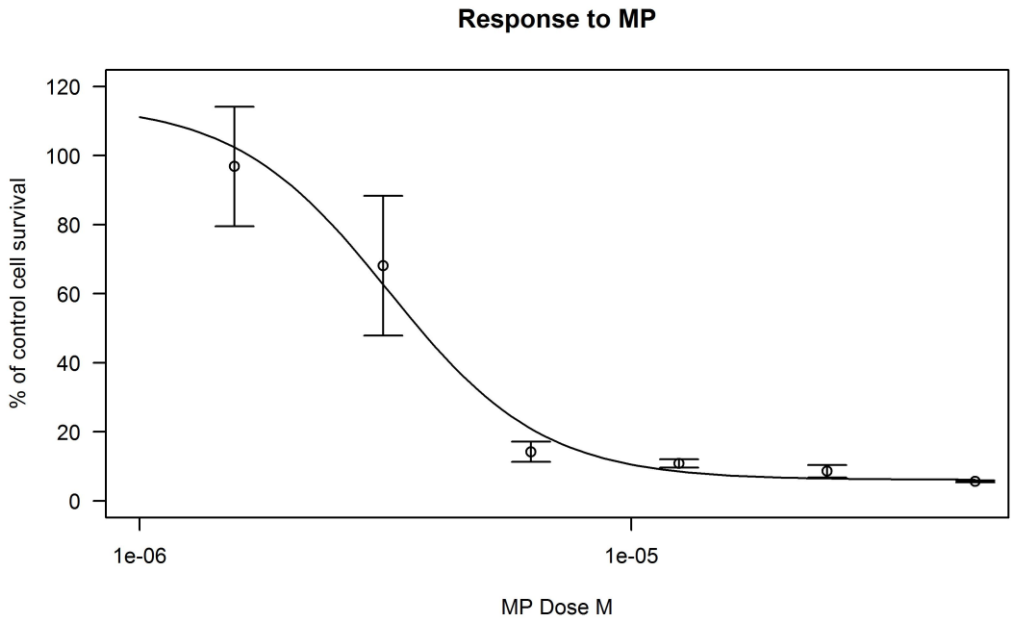
MOLT-4 cells have endogenous MTAP activity whereas Jurkat cells lack endogenous MTAP activity which affects metabolism and sensitivity to thiopurines (Coulthard *et al.*, 2011). Therefore the model was based on data collected in MOLT-4 cells. The sensitivity of MOLT-4 cells to MP was tested to ensure that the cell line was appropriate to produce data for a model of thiopurine metabolism in sensitive cells. AZA caused cell death in a dose dependent manner with less than 10% cell survival at a dose of 100 µM AZA (Figure 2.4 a). MP caused cell death in MOLT-4 cells at concentrations between 50 µM and 0.4 µM (Figure 2.4 b). The mean ED 50 for MP was 2.9 µM (SD 0.78), compared to a mean ED 50 of 6.0 µM (SD 0.44) for AZA from 3 biological repeats. In MOLT-4 cells MP was significantly more toxic than AZA ($P< 0.01$).

2.3.3 Sensitivity of MOLT-4 cells to a combination of MP and allopurinol

Combination studies of MP with allopurinol were done on MOLT-4 cells to determine how the addition of allopurinol affects the cytotoxicity of MP. When 1 mM allopurinol was added to MP (10 times the highest dose of MP) there was a large reduction in the toxicity of MP. The ED 50 increased more than 10 fold from 2.9 µM to 43.0 µM, this was a highly significant ($P< 0.001$) decrease in sensitivity. In contrast 100 µM allopurinol appeared to increase MOLT-4 sensitivity to MP, the ED 50 dropped to 1.0 µM, this was a non-significant increase in sensitivity ($P=0.980$). With 10 µM allopurinol the ED 50 of 6-MP was slightly lowered to 2.1 µM, this difference was not statistically significant ($P= 0.999$) (Table 2.2 and Figure 2.5).



a



b

Figure 2.4 Cell survival curves showing sensitivity of MOLT-4 cells to thiopurines. Points are means, error bars 1 SD and curves 4 parameter logistic curves based on 3 repeats determined with DRC package in R **a** AZA **b** MP.

Drug	MP Mean (SD) n=3	MP plus 10 µM allopurinol Mean (SD) n=3	MP plus 100 µM allopurinol Mean (SD) n=3	MP plus 1 mM allopurinol Mean (SD) n=3
Effective Dose 50 (ED50)	2.9e-06 (7.8e-07)	2.1e-06 (1.3e-06)	1.0e-06 (7.7e-07)	4.3e-05 (1.5e-05)
P value from Dunnett contrasts	Control not applicable	0.999	0.980	<0.001

Table 2.2 ED50 concentrations of Aza and MP and MP in combination with allopurinol in MOLT-4 cultured cells mean and SD (n=3) data processed using DRC in R

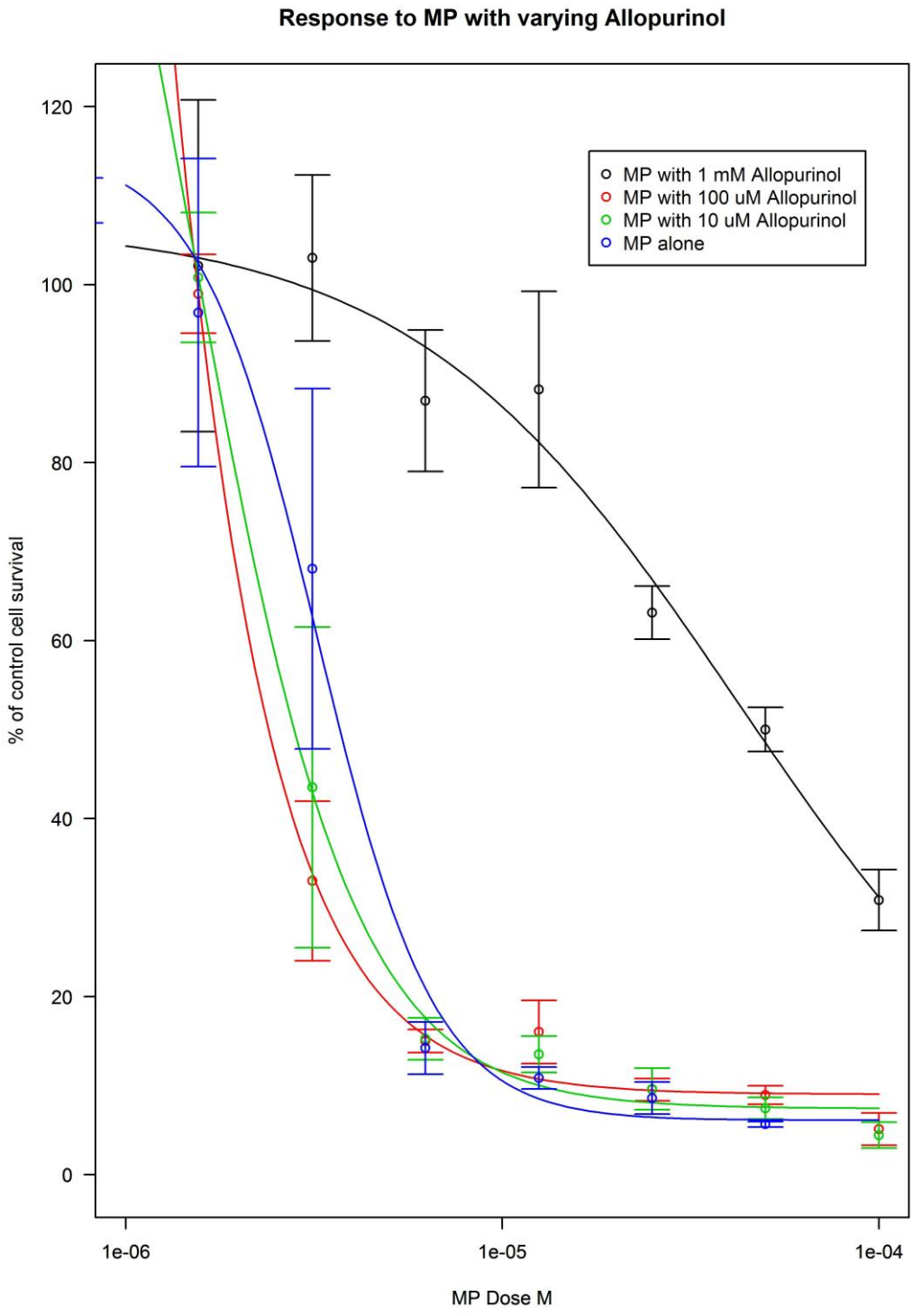


Figure 2.5 Cell survival curves showing sensitivity of MOLT-4 cells. Points are means (n=3), error bars 1 SD and curves 4 parameter logistic curves determined with DRC package in R **black**, MP plus 1 mM allopurinol, **red** MP with 100 µM allopurinol, **green** MP with 10 µM allopurinol and **blue** MP alone.

2.4Discussion

MOLT-4 cells show no reduction in cell survival in response to either allopurinol or oxypurinol, its active metabolite. The survival of MOLT-4 cells cultured with oxypurinol slightly increased in some cases. This is important to note given that subsequent work has considered the effect of combining allopurinol with MP on cell survival.

The effect of allopurinol on Jurkat MTAP +/+ cells was not significant and in Jurkat MTAP -/- cells allopurinol increased cell survival. Oxypurinol had no significant effect over three biological repeats on Jurkat MTAP -/- cell survival, although one repeat showed a significant reduction in cell survival with oxypurinol. The survival of Jurkat MTAP +/+ cells was reduced.

A serum concentration of 1.7 mM allopurinol has been shown to cause severe side effects and sometimes fatality in patients (Tam and Carroll, 1989). A more usual therapeutic allopurinol serum concentration is around 20 µM (Day *et al.*, 2007) suggesting that 1 mM allopurinol might have off target effects which may contribute to the variable cellular response to this concentration of drug. This concentration of allopurinol was difficult to dissolve, it is therefore possible that it was not fully dissolved and therefore in some repeats not at the correct concentration, also contributing to variability. This variability means that only very tentative conclusions may be drawn from this data.

Febuxostat was cytotoxic to all three cell lines; however in no cases was a plateau in cell survival reached making it difficult to determine ED 50 values. As the maximum concentration of febuxostat used was determined by its solubility no extension of the experiment could be made to determine ED50 values. It has previously been shown that allopurinol allows TNF related apoptosis-inducing ligand induced cell death in TNF-related apoptosis-inducing ligand resistant prostate cancer cell lines. This was shown to be due to a XO dependent reduction in C/EBP homologous protein levels (Yasuda *et al.*, 2008); C/EBP homologous protein is important to DNA damage induced apoptosis. It is possible that this or a similar effect due to XO inhibition may be the cause of the reduction in cell survival in cells treated with febuxostat.

Given the purpose of the experiments was to provide preliminary data for model building it would have been useful to assess the ED 50 of these drugs with

regard to XO inhibition as well as cytotoxicity. This would provide data to help fix the magnitude of XO inhibition by a given concentration of allopurinol in the computational model.

As previously reported MOLT-4 cells are sensitive to MP with a previously reported ED 50 value of 2.9 μM for MP after 72 hours, a very similar value to that observed here 3.21 μM (Fotoohi *et al.*, 2006a). This value is also in the reported range of maximum serum concentration in leukaemic patients. Values reported range from lower than detectable to around 600 ng/mL and 150 ng/mL is equivalent to 1 μM suggesting that these are clinically relevant values (Sulh *et al.*, 1986). MOLT-4 cells are slightly but significantly more sensitive to MP than to AZA.

Combining MP and allopurinol significantly reduced the cytotoxicity of MP when allopurinol was added at a much higher concentration than MP. When allopurinol was added at an equal or lower concentration than MP the sensitivity to MP was increased but not significantly. The effect of combining allopurinol and MP has not been previously studied in lymphocyte cell lines so no comparison can be made to previous data. The causes of these changes in the sensitivity to MP will begin to be examined later in this study, initially by examining the metabolite levels in cells treated with a combination of allopurinol and MP and also by testing possible mechanisms for these changes using a computational model of thiopurine metabolism.

2.5Conclusion

MOLT-4 cells are sensitive to MP and therefore a suitable model for the collection of parameters of MP metabolism to inform a computational model of MP metabolism.

The sensitivity of the MOLT-4 cells was different when MP was combined with allopurinol thus indicating that this system would also be suitable for exploring the effect of combing these two drugs using computational modelling.

Chapter 3. Development of a method for detecting thiopurine metabolites

3.1 Introduction

To develop a dynamic computational model of a biochemical system such as thiopurine metabolism, quantitative data are necessary to calibrate and validate the model. For this project some rate constants were derived from the literature however, not all of the kinetic parameters have been previously described. To estimate the remaining parameters, quantitative data about the changing concentration of as many as possible of the species being modelled need to be collected. Such data will allow estimates to be made of the kinetic parameters needed to produce these changes using software designed for this kind of estimation (for more details see Chapter 5). Simultaneous measurements need to be made of the concentrations of various thiopurine metabolites in cultured cells grown with MP. To this end a sensitive and reliable method capable of measuring a range of thiopurine metabolites is required.

A variety of methods for the detection of thiopurine metabolites have been developed, initially for use in a laboratory setting but later to permit analysis in a variety of cell types and body fluids. The first methods relied on paper chromatography (Maddocks and Davidson, 1975) but this has long been superseded by high pressure liquid chromatography (HPLC) (Breter, 1977) and increasingly by liquid chromatography mass spectrometry (LC-MS/MS) (Hofmann *et al.*, 2012; De Nicolo *et al.*, 2014). Methods for measuring thiopurine metabolites in a range of cellular compartments have been described: RBCs (Dooley and Maddocks, 1982; Lennard and Maddocks, 1983; Erdmann *et al.*, 1990; Lennard and Singleton, 1992; Weller *et al.*, 1995; Dervieux and Boulieu, 1998a), lymphoblasts (Dervieux *et al.*, 2002), isolated lymphocytes (Erdmann *et al.*, 1991), leukocyte DNA (Warren *et al.*, 1995; Cuffari *et al.*, 1996a; Wang and Wang, 2010b; Jacobsen *et al.*, 2012), plasma (Dooley and Maddocks, 1980; Andrews *et al.*, 1982; Lin *et al.*, 1991; Su *et al.*, 1999), whole blood (Pike *et al.*, 2001; Kirchherr *et al.*, 2013; Vikingsson *et al.*, 2013) and urine (Weller *et al.*, 1995). The most recent method uses LC-MS to detect TG and me MPR in isolated lymphocytes (De Nicolo *et al.*, 2014). HPLC has also been used to analyse the kinetics of MP metabolism in the lysate of cultured cells (Kalra *et al.*, 2007), a similar task to collecting data to estimate the

parameters of a dynamic model, suggesting that this may be an appropriate method to collect the data necessary for model parameter estimation.

3.1.2 Liquid chromatography and mass spectrometry

Previous methods of detecting thiopurine metabolites (Erdmann *et al.*, 1990; Lennard and Singleton, 1992; Dervieux and Boulieu, 1998a) have mostly used reverse phase HPLC. This is a method of separating chemicals by the relative affinities with which they bind a non-polar stationary phase (column packing) and the ability of a polar buffer to remove that affinity. Analytes are then detected by another means which in the case of thiopurine metabolites has commonly been by either UV absorbance (Lennard and Singleton, 1992; Dervieux and Boulieu, 1998a) or detection of fluorescence (Pike *et al.*, 2001; Vikingsson *et al.*, 2013). However more recent methods have used mass spectrometry (MS) to detect metabolites (Hofmann *et al.*, 2012; De Nicolo *et al.*, 2014).

Ion-exchange chromatography separates analytes based on ionic interactions between polar analytes and an oppositely charged polar stationary phase. It is commonly used for separation of oligonucleotides and therefore may be suitable for detecting thiopurine nucleotides. HPLC can be coupled to MS to detect analytes with a greater sensitivity than UV absorbance or fluorescence methods. MS detects metabolites based on their mass to charge ratio after ionisation and in the case of tandem MS on their fragmentation pattern when a charge is applied. A sample is ionised, in the case of electospray ionisation (ESI), by passage through a charged probe which passes a charge to the sample as it passes through. This is then vaporised and enters a first quadrupole where an initial separation by mass to charge ratio is made. These ions then enter a second quadrupole where they are fragmented by bombardment with argon or nitrogen gas in a vacuum, and form characteristically sized fragments. This allows further separation of the analyte mixture with the same initial mass to charge ratio, using the mass to charge ratio of these fragments in the third quadropole. The fragment of interest can then be quantified using a photo-multiplier based detection system. Recent advances in MS technology have led to the development of assays for thiopurine metabolites using this method. De Nicolo *et al.* have used the increased sensitivity to apply the assay to isolated lymphocytes (a less

abundant cell type than RBCs) (De Nicolo *et al.*, 2014), whilst Hoffman *et al* has used the ability of MS to uniquely identify many similar compounds to produce an assay capable of simultaneously measuring 11 thiopurine metabolites (Hofmann *et al.*, 2012). Both of these characteristics of LC-MS/MS make it an attractive method to develop to measure metabolites both to inform development of a computational model and as a novel clinical assay.

3.1.3 Measurement of thiopurine metabolites in clinical use

There have been several analyses of the effectiveness of thiopurine metabolite measurements at predicting the outcome of thiopurine therapy, some concluding that metabolite measurements are useful (Osterman *et al.*, 2006; Ooi *et al.*, 2007; Moreau *et al.*, 2014) but others do not (Konidari *et al.*, 2014). A positive correlation between TGNs in RBCs and the likelihood of a good outcome of treatment (Schmiegelow *et al.*, 1995; Dubinsky *et al.*, 2000; Cuffari *et al.*, 2001; Wright *et al.*, 2004; Ooi *et al.*, 2007) has been shown several times in both leukaemia and IBD. Also me MPR in RBCs, used as representative of methylated inosinic metabolites, has been shown to correlate positively with the likelihood of hepatotoxicity (Dubinsky *et al.*, 2000; Dubinsky *et al.*, 2002; Derijks *et al.*, 2004). The ratio between methylated metabolites and TGNs is used by some gastroenterologists to determine which patients should be treated with a combination of AZA and allopurinol. A high methylated to TGN ratio indicates that the patient may benefit from AZA and allopurinol combination and a reduction in this ratio is seen a sign that this treatment is likely to be successful (Sparrow *et al.*, 2005; Leung *et al.*, 2009).

Some of the lack of consistency in the assessments of the utility of thiopurine metabolite measurements to predict efficacy may be due to method-dependent variation in thiopurine metabolite levels as described in Chapter 1. It should also be noted that RBCs are not the target cells for thiopurine drugs but are chosen as the cell type for assessment of TGN levels largely due to their abundance. Thiopurines target immune cells and leukaemic blasts depending on the condition being treated. These target cell types are nucleated cells and therefore have IMPDH and are able to metabolise thiopurines, as described in Chapter 1. Mature RBCs are anuclear, lack IMPDH and therefore are not able to produce TGMP but rely on transport from their extra-celluar environment.

Measurements of TGNs in RBCs only indirectly reflect their production and levels in target cells and therefore their efficacy.

Despite these issues, several studies of the correlation between reported metabolite levels and outcome show that TGN measurement correlates with remission in IBD and leukaemia, indicating that metabolite measurement is a useful clinical tool (Schmiegelow *et al.*, 1995; Osterman *et al.*, 2006; Ooi *et al.*, 2007; Nguyen *et al.*, 2013). A more direct measurement of thiopurine metabolites in more-clinically-relevant cell types may prove a more sensitive indicator of clinical efficacy and would also be a useful research tool for improving understanding of the mechanisms of thiopurine action.

The aim of this part of the project was to develop an assay that would be able to detect as many thiopurine metabolites as possible accurately and with sufficient sensitivity to detect them in cell cultured cells. A subsidiary aim is to make the assay suitable for detecting a range of thiopurine metabolites in patient samples.

3.1.4 Aims

1. To develop a method to detect a range of thiopurine metabolites in cell culture cells using either HPLC or LC-MS/MS.
2. To show the sensitivity and reliability of such a method.
3. To show the applicability of such a method to cultured cells and patient blood samples

3.2 Methods

3.2.1 Preparation of standards for LC-MS/MS

TGMP, TGDP, TGTP, meTGMP, meTGDP, meTGTP, meTIMP, meTIDP, meTITP and TIMP were purchased from Jena Biosciences (Jena, Germany) as 10 mM solutions. MP, XMP and allopurinol were bought from Sigma (Sigma, Gillingham, UK) in powder form. Analytes purchased as powder were dissolved in 0.1 M NaOH to 50 mM. Dilutions of all analytes were then made in MilliQ filtered, deionised water.

For analysis of patient samples, a mixture of TGMP, TGDP,TGTP, meTGMP, meTGDP, meTGTP, meTIMP, meTIDP, meTITP and TIMP standards was used; a 100 µM stock solution of this mixture was made and stored at -20 °C. A standard curve was prepared by dilution of stock solution in water to 50 nM then eight serial dilutions from 50 nM to 0.159 nM. Quality controls (QCs) were prepared by the same method at concentrations of 30, 15 and 3 nM.

During method development and for analysis of initial cell culture samples, the mixture used for the standard curve also included MP, and allopurinol. Standards from 31.25 nM to 0.488 nM were prepared by serial dilution in water; QCs were also made by dilution in water to 62.5, 31.25, and 15.25 nM and in some cases 250 nM.

3.2.2 Preparation of patient samples for LC-MS/MS

Blood from patients receiving a variety of thiopurine drugs was sent to Newcastle in Ethylenediaminetetraacetic acid (EDTA) tubes. Within 24 hours of sampling, 4 mL of blood was diluted 1:1 with PBS then layered onto 8 mL Lymphoprep^{TM} under sterile conditions. This was then centrifuged for 30 min at 800 g without braking. The mononuclear cell layer, containing peripheral blood mononucleocytes (PBMCs), was removed and remaining RBC were lysed by mixing with 20 times the volume of RBC lysis buffer (0.15 M ammonium chloride; 0.01 mM potassium bicarbonate and 0.1 mM EDTA). After 10 minutes the mixture was centrifuged to obtain a cell pellet which was washed twice with PBS and stored at -80 ^{0}C. The washed cell pellet was resuspended in 200 µL water and sonicated on ice 3 times for 5 seconds each at amplitude 5. They were then centrifuged for 5 min in a microcentrifuge at 16,873 x g and the

supernatant transferred to a glass insert for analysis with 10 µL retained for protein measurement by Pierce bicinchoninic acid (BCA) assay.

From the RBC layer present after Lymphoprep centrifugation and mononuclear cell removal, three aliquots of 200 µL each of RBCs were removed and washed twice with 1 mL of PBS before being frozen at -80 ^{0}C. When defrosted, the RBCs were diluted 1:100 in MilliQ water then sonicated at amplitude of 5 for 10 s, centrifuged 16,873 x g for 5 minutes and the supernatant transferred to a glass insert for analysis with 5 µL retained for protein quantification by Bradford assay. The Bradford assay was used with RBC samples due to its suitability for use with samples containing haemoglobin, whereas the Pierce BCA assay is not suitable for these samples.

(Some samples were prepared by Dr. Sally Coulthard.)

3.2.3 Preparation of cultured cells for analysis by LC-MS/MS

Cells were grown as described in Chapter 2 and 5. Cell pellets of approximately 3 x 10^6 cells were stored at -80°C prior to analysis.

Cell samples were defrosted and resuspended in 200 µL of MilliQ filtered water. They were then sonicated on ice for 10 seconds at amplitude 4, centrifuged for 7 min in a microcentrifuge at 3000 g and the supernatant transferred to a glass insert for analysis; a 10 µL aliquot was retained for protein measurement by Pierce BCA assay.

3.2.4 Protein quantification by Pierce bicinchoninic acid and Bradford assays

The total protein in PBMCs and cell culture samples was measured using a Pierce BCA protein assay which is based on the Biuret reaction (reduction of Cu^{2+} to Cu^{1+} by protein) followed by chelation of BCA by the Cu^{1+} ions to produce a purple colour detectable colourimetrically at 562nm. The BCA reaction amplifies the signal so that a lower detection limit is possible than with a Biuret reaction alone.

The assay was performed in a 96-well plate. A standard curve made with 2mg/mL bovine serum albumin (Thermo Fischer) diluted to 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg/mL in deionised, filtered water was placed in four wells per standard and eight wells contain distilled water as a blank. 10 µL of standard, blank or appropriately-diluted cell lysate sample was added to each well and then 190 µL

of reaction solution made following the manufacturer's instructions was added. Incubation was for 30 min at 37°C. The plate was then read using a FLUOstar Omega Microplate reader (LabTech, Oretenberg Germany) at 562 nm.

Another assay which is more accurate for protein quantification in samples which include haemoglobin, the Bradford assay, was used to analyse the RBC lysate. The Bradford assay relies on the covalent bonding of hydrophobic parts of proteins to Coomassie Blue dye to produce a solution with an absorbance at 595 nm.

The assay was performed in a 96-well plate. A standard curve of 0.05, 0.1, 0.2, 0.4 and 0.6 mg/mL of bovine serum album (Thermo Fischer, Loughbourgh, UK) diluted in deionised, filtered water was prepared. 10 µL of standard, deionised, filtered water (blank) or diluted cell lysate was added to each well. 200 µL of Bradford reagent (BioRad, Hemel Hempsted, UK) diluted 1 in 5 was added to each well and incubated for 30 min at room temperature. The plate was read at 595 nm using a FLUOstar Omega Microplate reader (LabTech, Oretenberg Germany).

3.2.5 Liquid chromatography tandem mass spectrometry

This method was developed with help from Mr Philip Berry, particularly during the tuning stages and early development of the chromatography method. Some further development of the chromatography method and all runs collecting data for this project were carried out independently.

LC-MS/MS was carried out on a Shimadzu Prominence UFLC (Shimadzu, Kyoto, Japan) for chromatographic separation combined with an API4000 triple quadrupole LC-MS/MS (Applied Biosystems, California, USA) for tandem mass spectrometry (MS/MS) analysis.

A Clarity Oligo-WAX column (150mm x 4.6mm) and SecurityGuard Oligo-WAX column (4 x 3mm) both from Phenomenex (Cheshire, UK) maintained at 30°C were used with two ammonium acetate mobile phases of pH 8 (Buffer A) and pH 10.01 (Buffer B) for chromatographic separation of analytes. Mobile phases were prepared by weighing 1.5 g glacial acetic acid (ThermoFischer Scientific) and adding water. The pH was adjusted to pH to 8 or 10.01 with ammonia (ThermoFischer Scientific) and the final weight was adjusted to 500 g. The gradient system had a starting condition of 90% Buffer A followed by a 0.5

minute gradient to 80% Buffer A at 1.5 minutes. This was followed by a 2 minute gradient from 80% Buffer A to 100% Buffer B at 3.5 minutes with those conditions maintained until 13.5 minutes when the column was returned to 10% Buffer A in a 0.5 minute gradient. The flow rate was 0.7 ml/minute and a post-column flow splitter was utilised to divert 70% of mobile phase to waste to improve ionisation.

LC-MS/MS analysis with electrospray ionisation was performed in positive ionisation mode using nitrogen gas at the following optimised settings: curtain gas, 10; ion source gas 1, 40; ion source gas 2, 50; ion spray voltage, 5500; collision gas, 6; entrance potential, ionisation temperature, 400˚C. (Optimisation was performed by Mr Philip Berry and Dr Sally Coulthard for several analytes and by me with the help of Mr Philip Berry for the rest.) Optimisation of MS/MS parameters for all analytes was performed by selecting precursor ions and determining the four most prominent product ions. The three best of these were then further optimised for fragmentation and voltage parameters. Quantification of analytes was performed in multiple reaction monitoring (MRM) mode mass transitions and optimised MS/MS parameters are given in Table 3.1. Analyst® software v1.5 (AB SCIEX, Framingham, USA) was used for sample analysis, peak integration and analyte quantification.

Analyte	Retention Time (min)	MRM transition (*m/z*)	Declustering Potential (V)	Collision Energy (eV)	Collision exit potential (V)
TGMP	8.34	380.12 -> 168.2	61	25	10
TGDP	8.93	460.2 -> 168.1	56	33	12
TGTP	9.45	540.08 -> 168.0	81	33	12
meTIMP	6.05	379.03 -> 167.2	51	21	12
meTIDP	8.17	459.07 -> 167.2	61	23	12
meTITP	8.77	539.07 -> 167.2	81	29	5
meTGMP	7.57	394.03 -> 182.2	56	25	12
meTGDP	8.72	474.05 -> 182.1	71	25	12
meTGTP	9.37	554.04 -> 182.2	91	31	12
ATP	8.59	508.03 -> 136.2	86	41	14
GTP	8.91	524.13 -> 152.2	56	37	10
TIMP	8.18	365.07 -> 153.1	51	19	10
allopurinol	4.4	136.99 -> 110.2	71	29	8
thioguanine	5.74	168.12 ->134.0	61	31	26
mercaptopurine	6.55 or 8.19	153.02 -> 119.2	66	29	10

Table 3.1 Chromatographic and mass spectrometry detection characteristics of analytes.

3.2.6 Determination of variability

In order to determine the intra-day variability of the assay five injections of three QCs, one each at 3 nM, 15 nM and 30 nM, were made. The co efficient of variation of these five injections was determined (Mean/SD). This was repeated on five separate days in order to determine the inter-day variability.

3.2.7 Determination of matrix effect

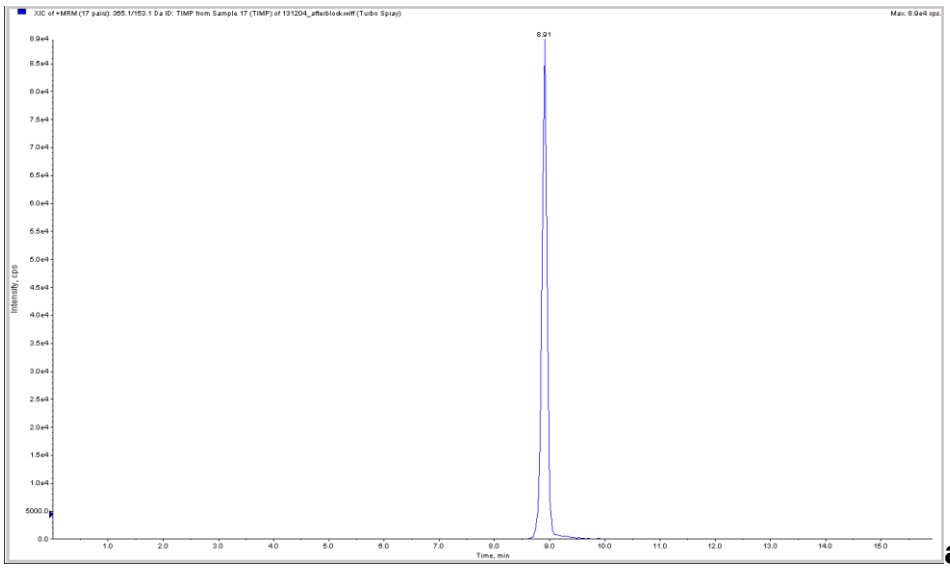
Matrix effect was determined by adding 200 µL of 3 nM, 15 nM and 30 nM QC to either pelleted PBMCs or RBCs obtained from healthy, untreated volunteers. The sample was then sonicated and centrifuged as described for patient and cell culture samples. The concentration of each analyte calculated from these samples was then compared to injections of the same QCs without cell lysate.

3.3 Results

3.3.1 Separation of purine nucleotides and analogues by LC-MS/MS

TIMP and XMP both have a molecular mass of 364 g/mol, ionic mass of 365.07 and produce a fragment to give the same Q3 mass, 153.1. The equipment was tuned to detect TIMP but when control cells (not grown with thiopurine drug) were assayed, a peak was evident in the TIMP mass channel at approximately the expected retention time for TIMP. This was due to XMP giving the same fragmentation pattern as TIMP, confirmed with XMP standard; however, it was possible to separate the two peaks by adjusting chromatography conditions whilst preserving the distinct peaks of the other metabolites. As shown in Figure 3.1 TIMP and XMP are distinguishable chromatographically but not by molecular mass or fragmentation pattern.

Also extra peaks were visible in the chromatograms for TGMP, TGDP and TGTP standards (Figure 3.2 a), these correspond to the retention times for meTIMP, meTIDP and meTITP. The mass of TGMP, TGDP and TGTP differ from meTIMP, meTIDP and meTITP respectively by only 1 the resolution of the mass spectrometer is 2, therefore it is likely that these extra peaks are meTIMP, meTIDP and meTITP.



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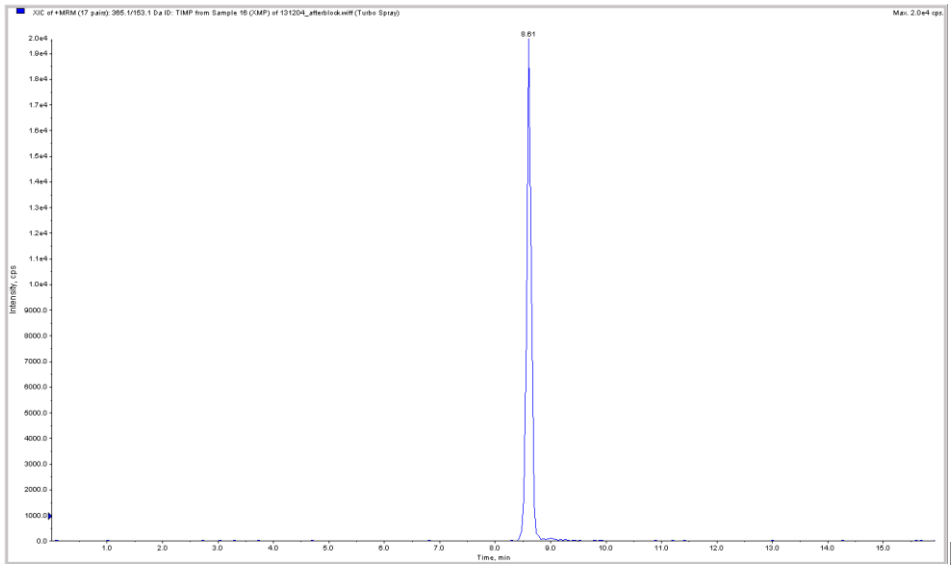

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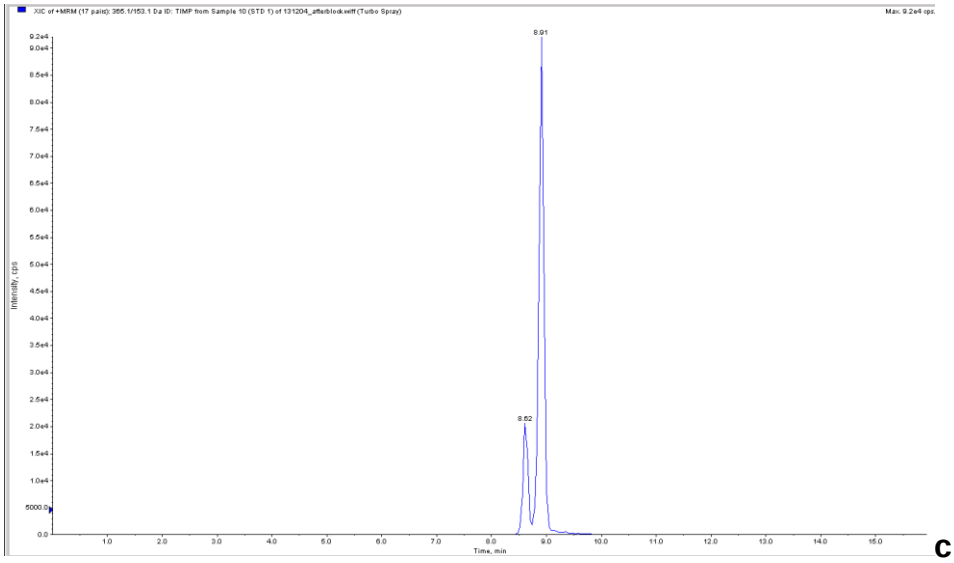


c

Figure 3.1 Chromatograms from Analyst **a** TIMP injected alone with 8.91 minutes retention time of XMP **b** XMP injected alone 8.61minutes retention time and **c** TIMP and XMP injected together.

3.3.2 Variability of standards analysed by LC-MS

Intra-day and inter-day variability was assessed for a total of 16 analytes; endogenous purines (adenosine triphosphate (ATP), GTP and XMP), a XO inhibitor (allopurinol, which is also a purine analogue) and thiopurine metabolites (AZA, MP, meTGTP, meTGDP, meTGMP, TGTP, TGDP, TGMP, meTITP, meTIDP, meTIMP, TG and TIMP). This assessment originally took place between October and November 2013. However the results of these runs were inadequate and as briefly discussed below, these experiments were re-run using a revised protocol in May and June 2014. These results are discussed in more detail.

Food and Drug Administration (FDA) guidelines for bioanalytical method validation (Administration, 2001) propose an inter-day covariance of less than 15% for the QCs and less than 20% for the lowest QC is acceptable for clinical assays. On initial analysis in 2013, metabolites showed an inter-day variability of less than 15% at all QC levels (31.25 nM, 62.5 nm, 125 nM and 250 nM). However allopurinol and meTITP had higher variability at 31.25 nM QC of 28% and 18% respectively. The preferred upper limit of variability for an assay is 20% for the lowest QC so the lowest safe QC for allopurinol was 62.5 nM.

In 2013 coefficients of variance between days were frequently unacceptably high. This was particularly the case for allopurinol at the lowest QC concentration. Other metabolites also showed considerable variation between repeated injections of at least one QC on several days. However on many occasions the intra-day variability was well below the required level.

Most differences from expected value of QCs for each day were in the range of 10% to 15% however there were many examples which show a smaller difference from expected values in 2013. All analytes differed from the expected QC value by 2% or less from the expected values at least once. Despite this, many QCs showed differences from control of between 20% and 30% difference from control. This was unacceptably high. The most extreme example was meTITP which at 31.25 nM showed a difference from control of 36% with a calculated value of 20 nM.

Due to this high variability between runs the protocol was revised, buffer pH was controlled more precisely by taking account of temperature variation, inter-

day and intra-day assay variability was re-determined. The limit of detection (three times the baseline) for the majority of metabolites was 0.391 nM whilst meTGDP and TIMP were detectable to 0.195 nM (Table 3.6). The lower limit of quantification is the lowest level at which a value can be assigned to a sample. According to FDA guidelines (FDA, 2001) this should be 5 times the baseline, however as for some analytes this was below the lowest sample used or at a level where samples showed high variability the lowest used concentration which showed less than 15% variability was used. The lower limit of quantification was 0.391 nM for most metabolites. For meTGMP, meTIMP, meTIDP and TIMP the lower limit of quantification was 0.781 nM. These values were adequate for the detection of thiopurine metabolites in patient samples as (Table 3.4) The upper limit of the linear range described is the maximum standard injected, 50 nM; this also appears adequate for quantifying metabolites in patient samples (Table 3.4). However, a higher standard curve was used for analysing cell culture samples (Table 3.4 and Figure 3.2b), and the reliability of the assay was acceptable to 250 nM in the earlier analysis. QCs up to 1000 nM were injected where necessary and these showed less than 20% difference from expected values on back calculation. The correlation coefficient was over 99% for all of the reanalysed analytes except TIMP, showing that the quantification of samples will be accurate in the range described.

As shown in Table 3.2, the intra-day variability was below 5% for the 30 and 15 nM QCs for all analytes except TIMP and below 10% at the 3 nM QC for all but TIMP. The interday variability was below 10% for all analytes except TIMP and below 15% for all analytes except TIMP. These low values for variability measures show the assay to be repeatable and, therefore, appropriate to analyse samples for future research. Nevertheless, the higher variability for TIMP show that data collected for TIMP in the 0.781 nM to 50 nM range should be viewed with caution and have not been included in the analysis of patient samples. TIMP was present at high concentrations in cell culture samples, and since the earlier assays had acceptable intra and interday variability at higher TIMP concentrations (250 nM to 31.25 nM), data from TIMP have been included in the analysis of samples from cell culture.

	Analyte									
	meTGMP	**meTGDP**	**meTGTP**	**meTIMP**	**meTIDP**	**meTITP**	**TGMP**	**TGDP**	**TGTP**	**TIMP**
LOD*a* (pmol/ml)	0.391	0.195	0.391	0.391	0.391	0.391	0.391	0.391	0.391	0.195
LLOQ*b* (pmol/ml)	0.781	0.391	0.391	0.781	0.781	0.391	0.391	0.391	0.391	0.781
Linear Range (pmol/ml)	0.781–50	0.391–50	0.391–50	0.781–50	0.781–50	0.391–50	0.391–50	0.391–50	0.391–50	0.781-50
Slope*c*	3216.98	2034.70	1340.44	1645.89	1722.30	1170.52	2107.93	1942.62	2392.71	1694.54
Intercept*c*	237.43	-10.48	-21.70	241.88	10.01	-91.04	-109.03	-533.24	-501.83	-1695.1
Correlation Co-efficient (r^2)	0.9998	0.9998	0.9997	0.9996	0.9996	0.9996	0.9996	0.9997	0.9995	0.9831
Intra-day High QC*d*	2.1	1.9	2.1	2.0	1.8	2.2	2.3	1.6	1.7	1.8

(%RSD)										
Intra-day Medium QC_d (%RSD)	3.1	2.5	4.5	3.4	2.3	3.6	3.5	2.4	1.9	2.3
Intra-day Low QC_d (%RSD)	6.0	6.2	7.1	8.3	5.1	5.7	5.7	5.2	2.8	5.1
Inter-day High QC_e (%RSD)	4.9	4.6	5.6	3.8	4.0	6.3	6.1	3.0	5.1	7.8
Inter-day Medium QC_e (%RSD)	5.3	5.6	4.7	5.9	4.0	4.8	7.1	4.3	7.1	12.5
Inter-day Low QC_e (%RSD)	8.5	8.0	4.8	7.6	7.2	7.7	11.6	14	6.2	20.7

Table 3.2 (previous 2 pages) Showing the within and between day variability of meTGMP, meTGDP,meTGTP, meTIMP, meTIDP, meTITP, TGMP,TGDP, TGTP and TIMP as assayed in January and May 2014

[a] Limit of detection (S/N = 3; n = 5); [b] Lower Limit of Quantification (S/N = 5 + replicate CV<15%; n = 5); [c] Calibration curves (y=ax+b); [d] Intra-day, n = 10; [e] Inter-day, n =5

3.3.3 Matrix effect

The effect of cell lysate on the detection of the nine key metabolites for the assay of patient samples was generally quite a large suppression (Table 3.3). However levels of a few metabolites showed amplification in cell lysate compared to water. Part of the difference in signal may be down to conversion between different phosphate forms of the nucleotides, particularly di and tri phosphates as previously described (Vikingsson *et al.*, 2013).

In PBMC lysate meTGTP showed almost complete loss of signal, meTGDP also showed a large loss of signal in PBMC lysate and meTGMP showed a gain of signal in PBMC lysate. This was different from the situation in RBC lysate in which meTGTP showed a large signal gain, meTGDP behaved similarly to in PBMC lysate and meTGMP lost some signal in RBC lysate. Overall the meTGNs lost around 40% signal in PBMCs lysate but only around 10% in RBC lysate.

In PBMC lysate all methylated thioinosine metabolites showed signal suppression, this was greatest for meTITP and least in meTIMP with the sum being suppressed by approximately 60%. In RBC lysate meTITP signal was amplified by around 50% while meTIDP and meTIMP signals were both suppressed. This leads to an overall suppression of around 10% signal.

Almost all TGTP signal was lost in PBMC lysate but was amplified by around 50% in RBC lysate. TGDP signal was lost in both PBMC lysate and RBC lysate. TGMP signal was amplified in both RBC and PBMC lysate. Overall signal loss of TGNs was seen in PBMC lysate. Whereas in RBCs signal loss for TGNs was seen at the middle concentration assessed but signal gain was seen at low concentration and no effect at high concentration. This is probably due to the matrix effect in RBCs being small compared to the variability of TGNs, possibly due to oxidation in cell lysate.

Analyte	Matrix Effect PBMC			Matrix Effect RBC		
	3 nM	**15 nM**	**30 nM**	**3 nM**	**15 nM**	**30 nM**
MeTGTP	-101%	-101%	-100%	125%	77%	48%
MeTGDP	-79%	-80%	-82%	-72%	-73%	-52%
MeTGMP	6%	78%	71%	-40%	-10%	-11%
Sum MeTGN	-50%	-35%	-39%	-9%	-1%	-5%
MeTITP	-67%	-91%	-95%	58%	53%	17%
MeTIDP	-30%	-57%	-63%	-51%	-66%	-47%
MeTIMP	-8%	-21%	-24%	-32%	-11%	-16%
Sum MeTIN	-34%	-56%	-60%	-10%	-10%	-16%
TGTP	-90%	-100%	-100%	78%	44%	28%
TGDP	-61%	-94%	-95%	-39%	-70%	-39%
TGMP	137%	85%	52%	28%	1%	11%
Sum TGN	-3%	-43%	-48%	24%	-9%	0%

Table 3.3 Matrix effect of PBMC and RBC cell lysate on nine key metabolites

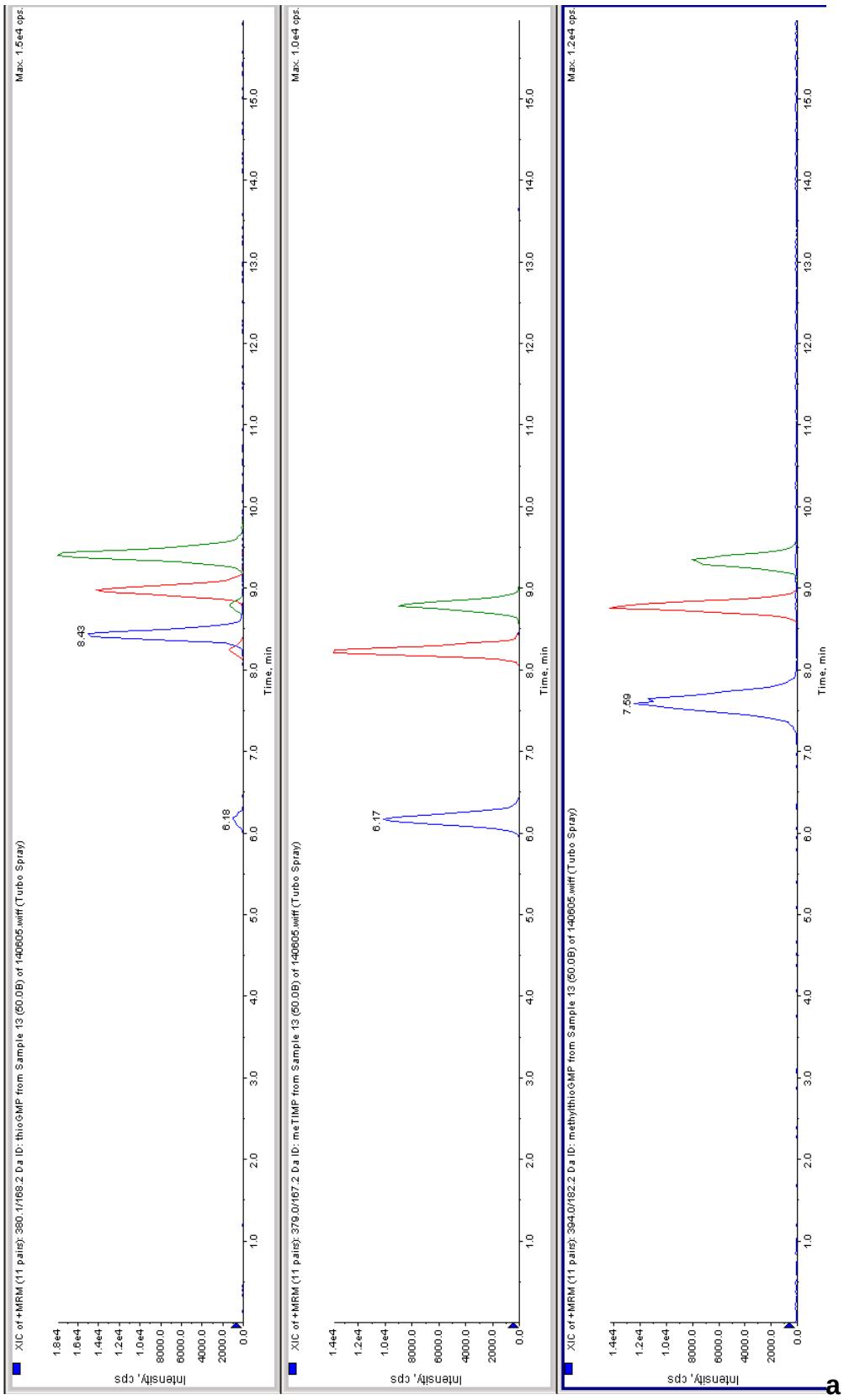
3.3.4 Analysis of patient and cultured cell samples

As shown in Table 3.4 and Figure 3.2 b and c, meTGMP, meTGDP, meTGTP, meTIMP, meTIDP, meTITP, TGMP, TGDP and TGTP were detected in samples from patients taking thiopurine drugs. They were detectable and quantifiable in both RBCs and PBMCs. There was a wide range of levels of most metabolites detected in cells from different patients; however, meTIDP, meTITP and TGTP were not detectable in any PBMC samples.

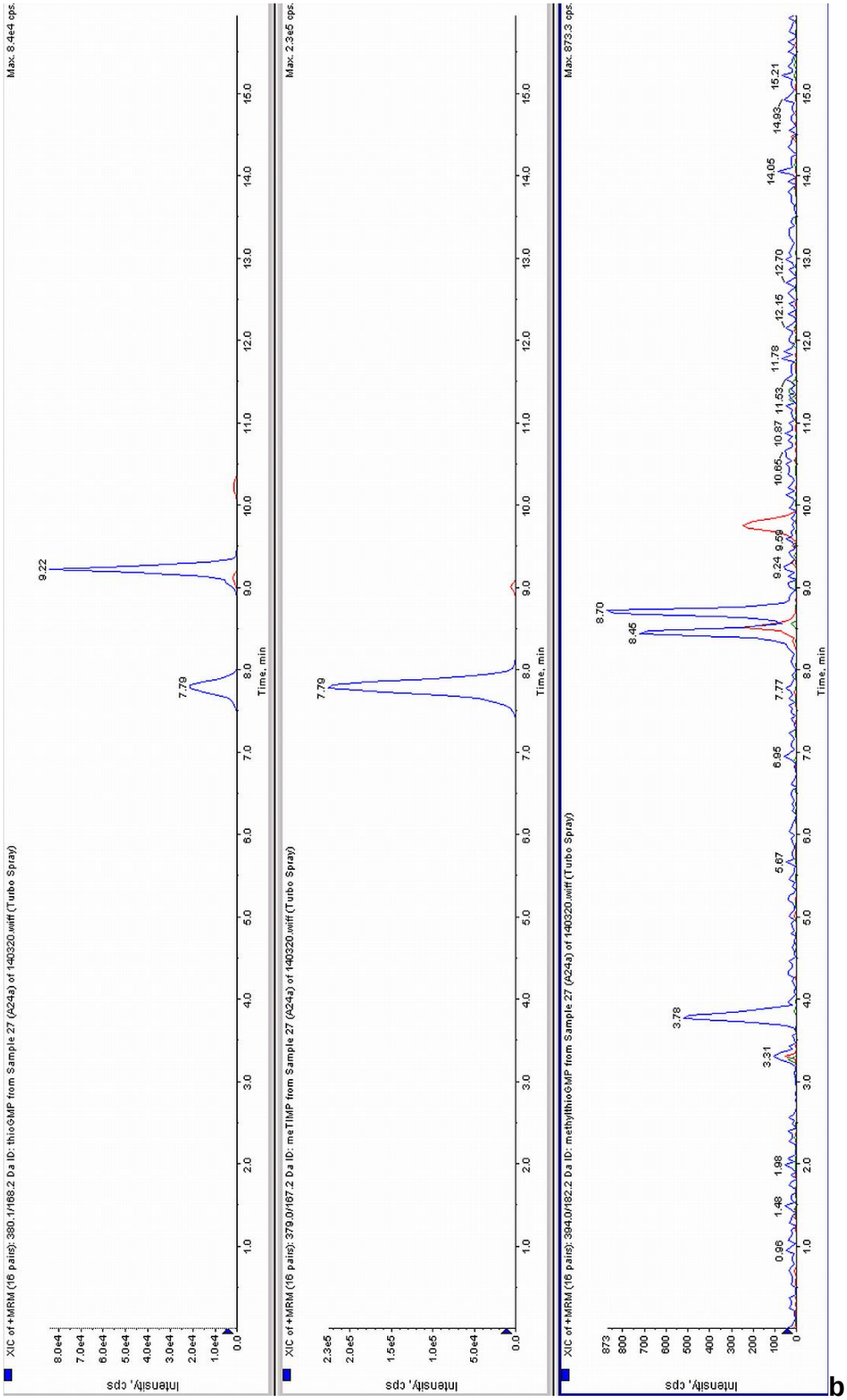
All analytes were detectable in MOLT-4 cells (Table 3.4 and Figure 3.2 a) cultured with 10 µM MP for 24 hours. They were found at higher concentrations than those seen in patient samples. meTIMP and meTDP and TGMP are around 1000 times higher than those detected in patient samples. Further discussion of the levels of MP metabolites detected in cultured cells is made in Chapter 5.

Metabolite	Range PBMCs pmoles/mg protein	Range RBCs pmoles/mg protein	Range in MOLT-4 cells pmoles/mg protein
MeTGMP	0.000-0.693	0.000-0.366	1.854-12.410
MeTGDP	0.000-0.159	0.000-0.863	1.318-9.643
MeTGTP	0.000-0.130	0.000-1.396	0.692-3.912
MeTIMP	0.000-0.569	0.000-2.240	1620.723-3381.285
MeTIDP	0	0.000-15.428	58.582-194.703
MeTITP	0	0.000-6.390	2.264-14.433
TGMP	0.228-1.909	0.000-1.138	164.767-814.253
TGDP	0.000-0.725	0.000-2.477	8.234-63.507
TGTP	0	0.000-3.615	1.100-32.856

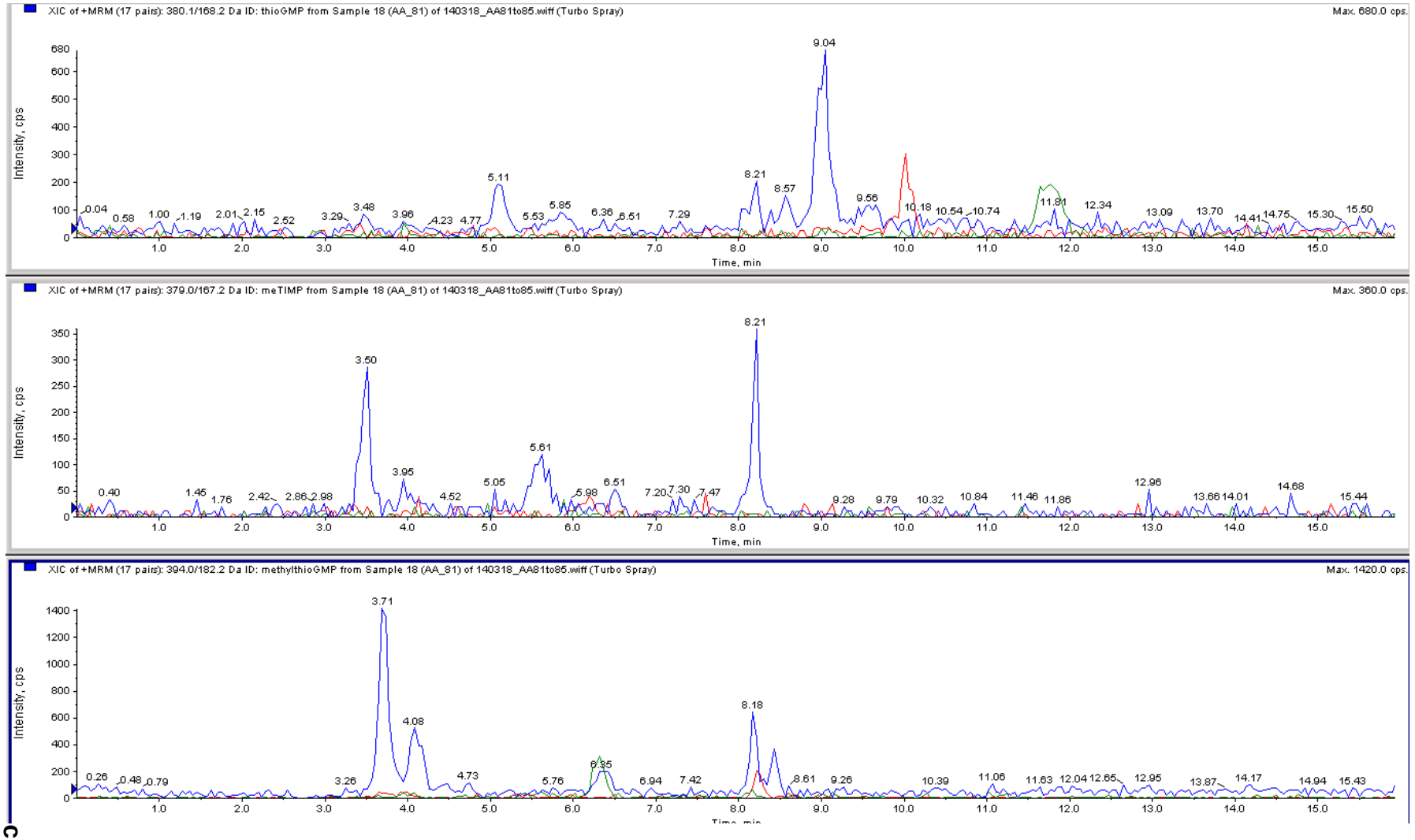
Table 3.4 The concentrations of various analytes detected in PBMCs and RBCs from10 samples from 10 patients on low dose azathioprine plus allopurinol and from cells 6 samples of MOLT-4 cells grown with 10 µM MP for 24 hours

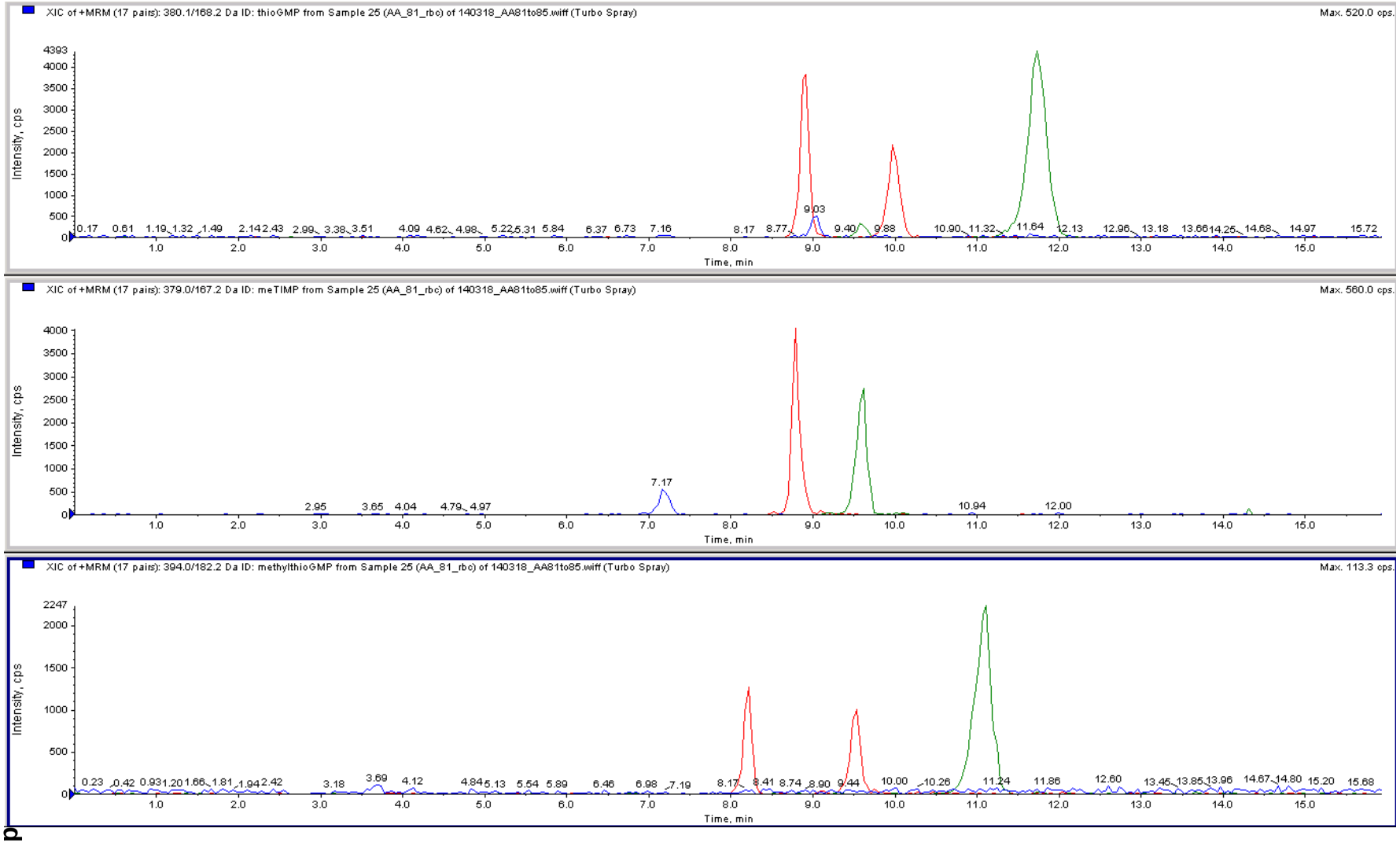

XIC of +MRM (11 pairs): 380.1/168.2 Da ID: thioGMP from Sample 13 (50.0B) of 140605.wiff (Turbo Spray)
Max. 1.5e4 cps.
8.43
6.18
Intensity, cps
Time, min
XIC of +MRM (11 pairs): 379.0/167.2 Da ID: meTIMP from Sample 13 (50.0B) of 140605.wiff (Turbo Spray)
Max. 1.0e4 cps.
6.17
Intensity, cps
Time, min
XIC of +MRM (11 pairs): 394.0/182.2 Da ID: methylthioGMP from Sample 13 (50.0B) of 140605.wiff (Turbo Spray)
Max. 1.2e4 cps.
7.59
Intensity, cps
Time, min

a


XIC of +MRM (16 pairs): 380.1/168.2 Da ID: thioGMP from Sample 27 (A24a) of 140320.wiff (Turbo Spray)
Max. 8.4e4 cps.
9.22
7.79
Intensity, cps
Time, min
XIC of +MRM (16 pairs): 379.0/167.2 Da ID: meTIMP from Sample 27 (A24a) of 140320.wiff (Turbo Spray)
Max. 2.3e5 cps.
7.79
Intensity, cps
Time, min
XIC of +MRM (16 pairs): 394.0/182.2 Da ID: methylthioGMP from Sample 27 (A24a) of 140320.wiff (Turbo Spray)
Max. 873.3 cps.
8.70
8.45
9.24
9.59
10.65
10.87
11.53
11.78
12.15
12.70
14.05
14.93
15.21
3.78
3.31
0.86
1.48
1.98
5.67
6.95
7.77
Intensity, cps
Time, min

b


XIC of +MRM (17 pairs): 380.1/168.2 Da ID: thioGMP from Sample 18 (AA_81) of 140318_AA81to85.wiff (Turbo Spray)
Max. 680.0 cps.
Intensity, cps
Time, min
XIC of +MRM (17 pairs): 379.0/167.2 Da ID: meTIMP from Sample 18 (AA_81) of 140318_AA81to85.wiff (Turbo Spray)
Max. 360.0 cps.
Intensity, cps
Time, min
XIC of +MRM (17 pairs): 394.0/182.2 Da ID: methylthioGMP from Sample 18 (AA_81) of 140318_AA81to85.wiff (Turbo Spray)
Max. 1420.0 cps.
Intensity, cps
Time, min


XIC of +MRM (17 pairs): 380.1/168.2 Da ID: thioGMP from Sample 25 (AA_81_rbc) of 140318_AA81to85.wiff (Turbo Spray)
Max. 520.0 cps.
Intensity, cps
Time, min
XIC of +MRM (17 pairs): 379.0/167.2 Da ID: meTIMP from Sample 25 (AA_81_rbc) of 140318_AA81to85.wiff (Turbo Spray)
Max. 560.0 cps.
Intensity, cps
Time, min
XIC of +MRM (17 pairs): 394.0/182.2 Da ID: methylthioGMP from Sample 25 (AA_81_rbc) of 140318_AA81to85.wiff (Turbo Spray)
Max. 113.3 cps.
Intensity, cps
Time, min

d

Figure 3.2 (Previous 4 pages) Chromatograms from Analyst: top panel TGMP approximately 8.4 minutes (**blue**), TGDP approximately 9.0 minutes (red) and TGTP approximately 9.5 minutes (**green**); middle panel, meTIMP approximately 6.2 minutes (**blue**), meTIDP approximately 8.2 minutes (**red**) and meTITP approximately 8.8 minutes (**green**); lower panel meTGMP approximately 7.6 minutes (**blue**), meTGDP approximately 8.8 minutes (**red**) and meTGTP approximately 9.2 minutes (**green**) **a** 50 nM Standards in water **b** MOLT-4 cells treated with 10 µM MP for 24 hours **c** PBMCs from a patient treated with low dose AZA and allopurinol, **d** RBCs from the same patient.

3.4 Discussion

Previously available assays for thiopurines were not able to detect a sufficient range of metabolites to collect the data needed to estimate the parameters for the computational model as they generally only measured TGNs and meMP (Lennard, 1987; Dervieux and Boulieu, 1998c; De Nicolo *et al.*, 2014). Hoffman published a more suitable method capable of detecting 11 metabolites (Hofmann *et al.*, 2012) but only after this project was well under way. Most of these previous methods used HPLC to detect TGNS and methylated metabolites. However due to the processing steps involved in these methods they are unable to distinguish between TGMP, TGDP and TGTP or between different methyl thioinosine metabolites and are unable to detect the methyl TG metabolites and TIMP, all of which would ideally be measured to provide data to inform the computational model. These previous methods are also of insufficient sensitivity as they were generally developed to detect metabolites in RBCs, an abundant cell type thus facilitating access to lysates with high concentrations of metabolites.

An initial HPLC assay was developed as part of this project which was able to assay more metabolites than most previously published methods and reduced the pre-analytical processing. However it was insufficiently sensitive and reliable to detect all the thiopurine metabolites in cell culture samples or to be used as a clinical assay.

For the LC-MS/MS assay developed during this project to detect analytes, the only processing of samples was cell lysis, in the case of cell culture cells, and cell separation and lysis in the case of patient samples. There have been several recently-developed methods for assaying thiopurine metabolites using LC-MS/MS (Hofmann *et al.*, 2012; De Nicolo *et al.*, 2014). Our LC-MS/MS assay was between 100 and 1000 times more sensitive than our earlier HPLC assay. The method recently developed by Hoffman (Hofmann *et al.*, 2012) was able to detect a range of thiopurine metabolites however it was only optimised for use with RBC samples and pre-processed with EDTA and DTT. More recently a method optimised for use with lymphocyte samples and with a similar sensitivity to the method described here was developed (De Nicolo *et al.*, 2014). However this method only detects thioguanosine and me MPR. This relies on processing of samples by phosphatase which reduces the number of

individually identifiable metabolites. This low number of metabolites detected makes it unsuitable for use in collecting data to inform a computational model. Whereas the method developed for this project was sufficiently sensitive to be used to detect metabolites in lymphocytes from patients as well as able to detect a wide range of analytes making it suitable for data collection for model calibration.

Other recently developed assays have found, similarly to this assay, that cell lysate causes suppression of some analytes and amplification of others (Hofmann *et al.*, 2012; De Nicolo *et al.*, 2014).

As the various phosphate forms of the metabolites have been shown to be considerably variable in cell samples (Vikingsson *et al.*, 2013) it was decided later in this project that for the purposes of using data, collected using this LC-MS/MS method, for computational model building the mono, di and triphosphate forms of each metabolite group were treated as one species in the model.

It was also decided that the quality of data collected about ATP and GTP was insufficiently reliable to be used to produce a model including these endogenous purines.

It should be noted that metabolite levels in RBCs, PBMCs and cultured cells differ. This may suggest that the modelling of MP metabolism in cultured cells may not be relied on to describe the situation in patients. These differences may also reflect the fact that the cultured cells were treated with MP and the patients who provided samples for analysis were treated with a combination of AZA and allopurinol. However the magnitude of the difference between cultured cells and patient samples is large especially for TGMP and therefore seems likely to reflect other differences such as a lack of first pass metabolism by the liver in cultured cells. Given that it is difficult to study drug metabolite levels in detail in patients this is a difference which must be acknowledged but does not stop the use of data collected in cultured cells.

3.5 Conclusions

A method of detecting 9 thiopurine metabolites in patient samples and 16 thiopurine metabolites or endogenous purines in cell culture samples with slightly less reproducibility at low concentrations has been successfully developed. This method has begun to be used to investigate the difference between RBC and lymphocyte metabolite levels in patients. This shows promise for further investigation which may help to optimise clinical metabolite monitoring in the future. This method has also been used to produce measurements of metabolites in cell culture samples to inform a computational model (results described in Chapter 5).

In the future this method may be used for both further investigation of the mechanism of action of thiopurines *in vivo* and *in vitro*. Also after a study of correlation with clinical outcome it may be able to be used in routine treatment monitoring.

Chapter 4. Towards an integrated model of purine and thiopurine metabolism

4.1 Introduction

It was argued in the introduction to this thesis (Chapter 1) that an understanding of the complexities of thiopurine metabolism is best addressed using a systems level approach rather than focusing in detail on individual components. Previous attempts at computational modelling the mechanisms and effects of thiopurine metabolism have provided some insight.

Panetta *et al* (Panetta *et al.*, 2006) have built a mechanistic model of the effects of TGN incorporation into DNA on the cell cycle and fitted data from various cell lines with differing sensitivities to thiopurine drugs. The model produced suggested that thiopurine resistance developed due to the cells failing to enter apoptosis but with a reduced cycling rate. This was confirmed with gene expression data showing altered expression levels of genes involved in cell cycle and apoptosis control.

Fransson *et al* (Fransson *et al.*, 2006; Fransson *et al.*, 2007) used a mechanistic model of thiopurine metabolism to model the action of AZA at the whole body level in patients over a period of several months. However they had limited success in matching their models to the available data. Both of these studies indicate that a simpler system informed by more targeted data might present a more tractable solution.

An obvious first step in producing a computational model of thiopurine metabolism is to build on a pre-existing well characterized model of endogenous purine metabolism, a closely related system. Several models of endogenous purine metabolism have been developed.

4.1.2 Computational models of endogenous purine metabolism

An early example of modelling purine metabolism was reported by Franco (Franco and Canela, 1984), but at this time available computing power was a severe restriction on what could be feasibly modelled. They used a limited selection of the purine pathways in their network, and did not include DNPS or incorporation into DNA. A later more extensive model was created by Curto (Curto *et al.*, 1997; Curto *et al.*, 1998a; Curto *et al.*, 1998b; Vera *et al.*, 2007). Three versions of this model were developed using different types of reaction

kinetic schemes: 1) Generalised Mass Action (GMA), a system which uses power law representations of individual reaction rates; 2) s-system, a simplification of the GMA system which by combines inputs and outputs for each reaction allowing for easier analysis of the model and; 3) Complemented Michaelis–Menten kinetics, which uses reaction rate laws familiar to biochemists and pharmacologists such as Michaelis–Menten and Hill equations. GMA type power law equations were added to fill in the gaps where details of reactions were unavailable. Analysis of the three versions of the model showed that all of the systems were reasonably representative of the purinergic system provided that conditions remained within the bounds initially assumed by the modellers. However for larger variations to the baseline conditions of the model, for example large variations in levels of some enzymes, the GMA system proved most robust (Curto *et al.*, 1998b). A subsequent paper showed that the GMA model proved useful for analysing various clinically relevant conditions including Lesch-Nyans disease (HGPRT deficiency) and suggested possible future therapies for this condition (Vera *et al.*, 2007). The model by Curto *et al* (Curto *et al.*, 1998b) has been uploaded to a publicly accessible database of computational models, the BioModels database (Chen *et al.*, 2010).

Of note, the Curto model (Curto *et al.*, 1998b) has been used as a basis for a recent model of the action of Mizoribine, an IMPDH inhibitor (Thomas, 2010). This model extended the original model by Curto (Curto *et al.*, 1998b) by separating nucleotides into di, mono and tri phosphate forms. This may be useful but it is difficult to justify given the currently available data, experimental techniques and the variable nature of the ratios of these species as shown by the qualitative but not quantitative agreement of the newly collected data of the individual nucleotide levels. This paper reports a new scenario that can be simulated using the model by Curto (Curto *et al.*, 1998b) as a framework. However, it does not increase the depth of detail of the model sufficiently to allow conclusions to be drawn regarding the changes in nucleotide ratios as would be wished in a model which attempts to describe the effects of Mizoribine on GTP.

4.1.3 Aims

1. To produce a graphical representation of the Curto model (Curto *et al.*, 1998b) in Cell Designer (Funahashi *et al.*, 2003) to aid visualisation
2. To simulate the network using CoPaSi (Hoops *et al.*, 2006) to verify that it behaves as described (Curto *et al.*, 1998b)
3. To further develop this model to include the metabolism of thiopurine drugs and in particular their interactions with the XO inhibitor allopurinol.

4.2 Methods

4.2.1 SBML

In order to facilitate the building, analysis and exchange of computational biological models, various standards for model representation have been developed. Systems Biology Mark-Up Language (SBML) (Hucka *et al.*, 2003) is currently the most widely used for modelling intracellular processes and is a machine-readable language for representing quantitative models of biological systems. It is based upon the eXtensible mark-up language (XML) a pre-existing standard. In brief, a model is defined by: its component 'species' (reactants and products), quantities for their concentration or abundance; all the reactions in which they interact, mathematical descriptions of reaction kinetics, together with related quantitative data for rate parameters. SBML is continually developed and released as a series of Versions and Levels with functionality being updated in each release. SBML was designed to enable the easy exchange of models between groups and between different programmes used within groups. Once fully developed, a model can be analysed and simulated using a variety of SBML compatible programmes, such as CellDesigner and CoPaSi. The ability to exchange models is enhanced by the existence of a publically accessible database, BioModels (see below), which provides a curated repository of published models. These models are available for download in SBML format that can then be reused, modified or extended to allow further predictions to be made and further insights to be gained.

4.2.2 BioModels Database

BioModels (Chen *et al.*, 2010) is a partially curated database of mathematical models of biological systems from which previously published models can be downloaded in a variety of formats including SBML. The model of purine metabolism described in Curto *et al* (Curto *et al.*, 1998b) has the identifier "BIOMD0000000015-Curto1998_purineMetabol". It has passed validity checks by BioModels staff and is stored in the curated section of the database. The model was downloaded using Cell Designer (Funahashi *et al.*, 2003) (see below 4.2.3) in the L2 V1 SBML.

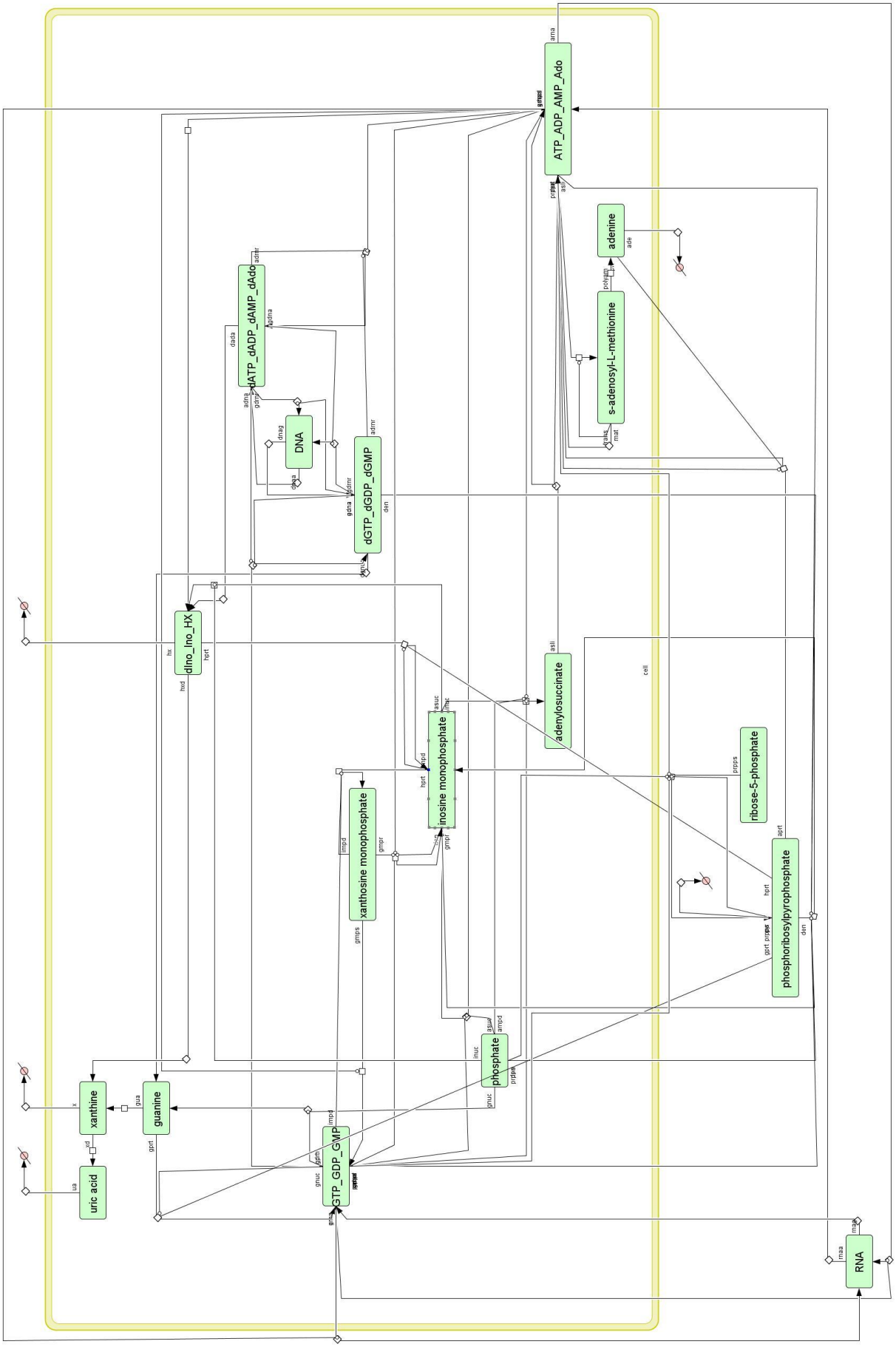

ATP_ADP_AMP_Ado
adenine
s-adenosyl-L-methionine
dATP_dADP_dAMP_dAdo
DNA
dGTP_dGDP_dGMP
dino_Ino_HX
adenylosuccinate
inosine monophosphate
xanthosine monophosphate
ribose-5-phosphate
phosphoribosylpyrophosphate
phosphate
xanthine
guanine
uric acid
GTP_GDP_GMP
RNA
cell

Figure 4.1 (Previous page) Cell Designer representation of the model of endogenous purine metabolism (Curto *et al.*, 1998b) downloaded from the BioModels database (Chen *et al.*, 2010).

4.2.3 Cell Designer 4.1

Cell Designer (Funahashi *et al.*, 2003) (version 4.1) is a software tool with a particularly well developed graphical user interface (GUI) which implements an extension to SBML, the Systems Biology Graphical Notation (SBGN) (Nicolas Le Novère *et al.*, 2009), to facilitate network visualisation. The extensions include additional XML to define positioning and provide defined styles to represent species such as genes, mRNAs, proteins and processes such as transcription and translation. Cell Designer is designed for the easy building, editing, simulating and graphical representation of dynamic computational models of biological systems. Cell designer was used to download the model of purine metabolism (Curto *et al.*, 1998b) from BioModels Database (Chen *et al.*, 2010) and to export it as an SBML (Hucka *et al.*, 2003) file for further editing in CoPaSi (Hoops *et al.*, 2006) (see below 4.2.4). Cell Designer was also used to produce graphical representations of models in SBGN, both those downloaded from BioModels and those created in CoPaSi can be imported to Cell Designer as SBML files.

4.2.4 CoPaSi 4.6.32

CoPaSi (Hoops *et al.*, 2006), derived from Complex Pathway Simulator, is a software tool designed to enable the building and analysis of dynamic biochemical models. It has a GUI that allows for the building of models without the need to directly write SBML but does not offer the network visualisation of CellDesigner. CoPaSi however, has more extensive mathematical functionality. It can be used to automatically create systems of ordinary differential equations (ODEs) from the rate laws specified for each reaction. CoPaSi also allows the model to be viewed as a matrix showing the stoichiometry of the defined reactions. CoPaSi supports SBML which allows the model to be moved easily between programmes. In this project the model of purine metabolism (Curto *et al.*, 1998b) was imported to CoPaSi in SBML format from Cell Designer. The model was then altered to vary the rates of various enzymes to replicate the manipulations described by Curto (Curto *et al.*, 1998b) as verification of the capacity of the model to replicate data from known clinical conditions (described below section 4.3.1). Time course simulations were run using the ODE deterministic (LSODA) simulator. Reactions and species were added in CoPaSi to the initial model to represent the metabolism of thiopurine drugs. Rate laws for new reactions were adapted from those described by Curto (Curto *et al.*,

1998b) for the corresponding reactions of endogenous purines. These models were exported to Cell Designer as SBML files in order to be visualised.

4.2.5 SBML Shorthand

An alternative to adding reactions to the existing model of purine metabolism (Curto *et al.*, 1998b) using the CoPaSi GUI, was to edit the model in SBML shorthand (Gillespie *et al.*, 2006). This is a text based simplification of SBML (Hucka *et al.*, 2003) which is easier for humans to read and write. The SBML shorthand code was translated to SBML using the web based conversion tool ('http://www.staff.ncl.ac.uk/d.j.wilkinson/software/sbml-sh/,') or by using the python based mod2sbml.py tool (Wilkinson, 2010). Unfortunately, a limitation of SBML shorthand is that it does not support the separate definition of rate laws outside their reactions, which is a feature allowed in SBML. As the Curto (Curto *et al.*, 1998b) model defines rate laws as separate functions outside the reaction, instead of as part of the reaction definition, it proved a time consuming process to edit the model by SBML shorthand and so final editing was done using the CoPaSi GUI. SBML shorthand did prove very useful for comparing different versions of the model to ensure that no errors were made.

4.2.6 Analysis of endogenous purine content of cell culture cells

The endogenous purine content, ATP, of MOLT-4 cells grown without any thiopurine drug treatment was analysed by LC-MS/MS in order to assess the utility of this data for estimating parameters of extensions to the model from Curto *et al* (Curto *et al.*, 1998b). Cells were grown and harvested as described in 3.2.3 and then analysed using the LC-MS/MS method described in 3.2.5; Q1 fragment of ATP was 508.032, Q3 was 136.200 collision energy were DP 86.000, EP 10.000, CE 41.000 and CXP 14.000 with a retention time of 9.03 minutes. This was compared to protein concentrations determined by the Pierce BCA assay described in 3.2.4.

4.3 Results

4.3.1 Replication of results from Curto et al (Curto et al., 1998b)

To assess how well the model stored in and downloaded from BioModels (Chen *et al.*, 2010) (graphical representation Figure 4.1) corresponded to the model published in “Mathematical models of purine metabolism in man” (Curto *et al.*, 1998b) the manipulations described in sections 4.3, 4.4 and 4.5 (Curto *et al.*, 1998b) of the Curto manuscript were recreated in CoPaSi. This was important because the model was not originally created in SBML and therefore may have been altered when it was translated to SBML before uploading to the BioModels database. Section 4.3 of Curto *et al* (Curto *et al.*, 1998b) described the effect of a 10-fold increase in phosphoribosyl pyrophosphate (PRPP) concentration. Section 4.4 (Curto *et al.*, 1998b) described the effect of a doubling of the rate constant of phosphoribosyl pyrophosphate synthase (PRPPS), to simulate PRPPS superactivity. Section 4.5 (Curto *et al.*, 1998b) described the effect of a 100-fold reduction in the rate constants of HGPRT activities with respect to HX and guanosine (HPRT and GPRT) to simulate HGPRT deficiency. As shown in Figure 4.2 a and b the model simulated in CoPaSi behaved as the models described in Curto *et al* (Curto *et al.*, 1998b) in response to a 10-fold increase in PRPP with regards to IMP and xanthine. Both initially increase and then decrease back to the baseline level. IMP increases from 98 to 109 µM in the model in CoPaSi. In the GMA model in the paper IMP is shown to increase slightly more, to around 119 µM. In both cases the concentration of IMP has reduced back to 98 µM by around 100 minutes. HX increases from 9.5 to 11.5 µM in the model version in CoPaSi and returns to its initial value after 100 minutes. The GMA version is shown to decrease slightly then to increase to slightly below 12 µM and to return to steady state, as reported by Curto *et al* (Curto *et al.*, 1998b). The fit is not perfect but is qualitatively similar in that both the downloaded and the reported models rapidly return to normal values for IMP and HX concentration after increasing the initial concentration of PRPP.

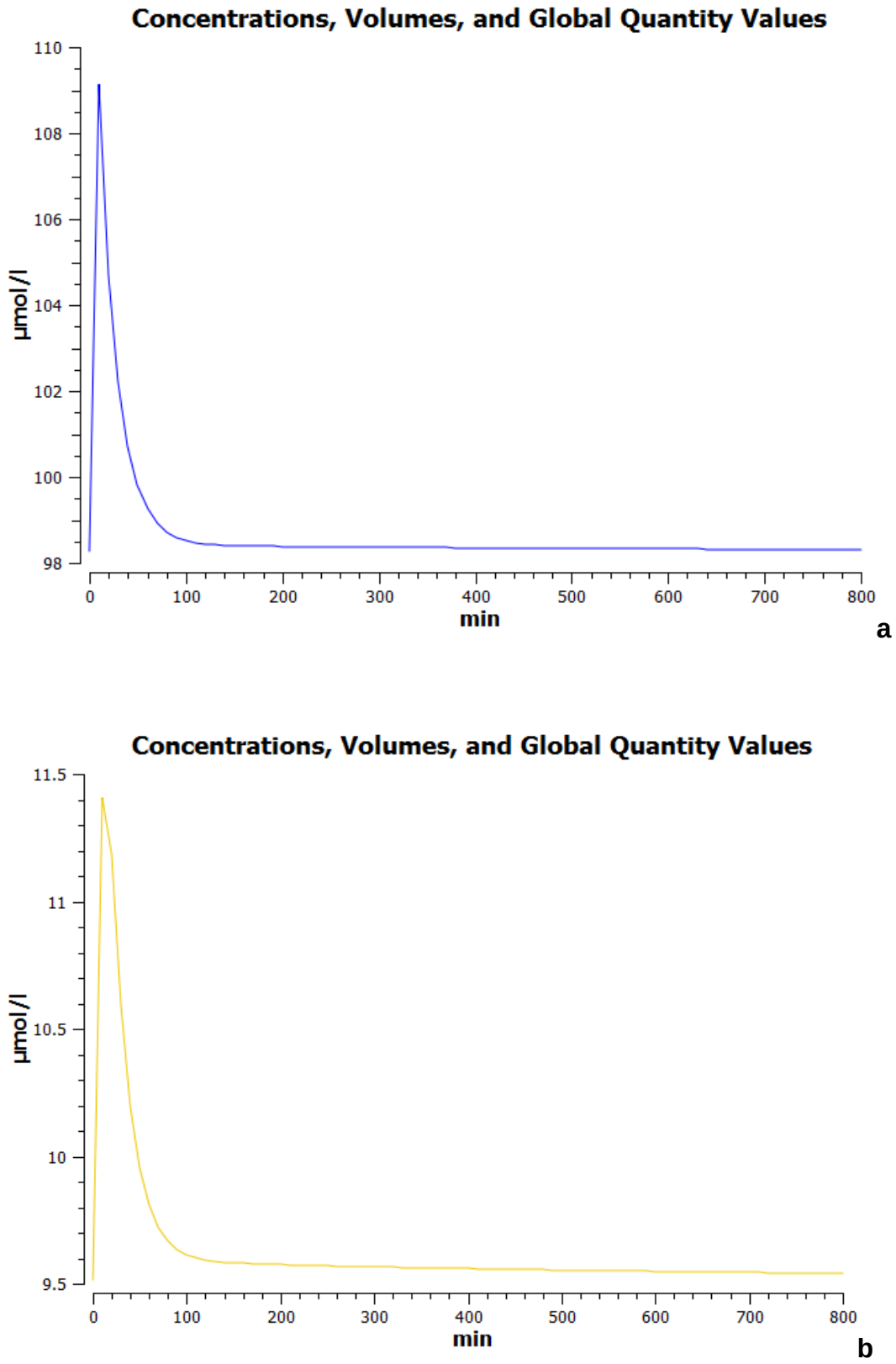


Figure 4.2 CoPaSi time course output showing the effect of a 10-fold increase in PRPP concentration at time 0 on **a** IMP in blue **b** Hx in yellow.

Increasing the activity of PRPPS two fold in a CoPaSi time course simulation (Table 4.1) had the same effect as described in Curto *et al* (Curto *et al.*, 1998b) (shown in Table 8 in Curto *et al* (Curto *et al.*, 1998b)). PRPP concentration, Vden flux, (the rate constant used by Curto *et al* to describe DNPS) Xa, (the designation in Curto *et al* of xanthine concentration) and Ua (the designation in Curto *et al* of UA concentration) all increased by the same amount in both the published version of the GMA model and the model as simulated in CoPaSi. This was generally less than the clinical data shown in Curto *et al* (Curto *et al.*, 1998b). Hx_ino_dino (a species in the model described by Curto *et al* (Curto *et al.*, 1998b) including HX, inosine and deoxyinosine concentrations), increased slightly less in the CoPaSi version than HX is described as doing; this is simply due to the fact that the authors of the paper disaggregated this value before reporting only HX to make it comparable to the reported clinical data. Both models slightly underestimate the clinical data with regards to changes to PRPP and UA but overestimate the effect of PRPPS activity on HX. However, as the model downloaded to CoPaSi was intended to replicate the model from Curto *et al* (Curto *et al*., 1998b), it would be inappropriate to expect the CoPaSi version of the model to compare better to clinical data than the model it was based on as no further model improvements or validation had been carried out.

	Initial Value	**CoPaSi Mean of 100 minutes**	**GMA Curto (Curto *et al.*, 1998b)**	**Clinical data as reported in Curto *et al (Becker et al., 1989; Jimenez et al., 1989; Curto et al., 1998b)***
PRPP µM	5.00	7.80	7.80	13-29
Vden µmoles / minute	2.39	4.70	4.70	4.7-12.4
Hx_ino_dino µM	10.00	40.30	41.00	30
Xa µM	5.00	14.80	15.00	10
Ua µM	100.00	131.00	131.00	300

Table 4.1 The outcome of increasing PRPPS activity 2-fold both in the model in CoPaSi and that previously reported by Curto *et al(Curto et al., 1998b)*

Reducing HGPRT activity increased the concentrations of Hx_ino_dino, Gua and UA (Table 4.2), the substrates for HPRT, GPRT and the product of an alternative reaction of Hx_ino_dino. This output compares well with Table 9 of Curto *et al* (Curto *et al.*, 1998b) and also the reported clinical data (last two columns of Table 4.2). The values reported by Curto *et al* (Curto *et al.*, 1998b) for a new steady state with reduced HGPRT activity were all matched by the mean value for 100 minutes when the model in CoPaSi is simulated as a time course with altered HGPRT activity. Due to the fact that the quantity of excreted metabolites (such as UA) accumulate in the downloaded model, the reactions which represent excretion create new species in the model and steady state cannot be reached as the model structure does not have outputs which can equal the inputs. For this reason steady state values cannot be analysed, so an alternative way of assessing these values was used; the mean value of each species over 100 minutes of model time were used to represent an approximation of steady state, given this the data match well.

	Initial Condition	CoPaSi GMA mean of 100 minutes	GMA Curto (Curto *et al.*, 1998b)	Clinical Data Shown by Curto *et al* (Seegmiller and Rosenbloom, 1967; Harkness, 1989; Fujimori et al., 1991; Curto et al., 1998b)
PRPP µM	5.00	7.14	7.10	-
Hx_ino_dino µM	10.00	69.81	70.60	71
Xa µM	5.00	22.55	22.50	15
Gua µM	5.00	9.62	9.00	-
UA µM	100.00	145.47	145.70	150
Vgua µmoles/minute	1.10	1.53	1.47	-
Vhxd µmoles/minute	1.23	4.35	4.40	-
Vxd µmoles/minute	2.30	5.27	5.30	7-14
Vhx µmoles/minute	0.05	0.44	0.44	0.45
Vx µmoles/minute	0.03	0.61	0.60	0.27
Vua µmoles/minute	2.30	5.27	5.30	7-14

Table 4.2 The effects of decreasing the rate constants of HPRT and GPRT 100-fold in CoPaSi compared to the original model data from the simulation in CoPaSi and from Curto *et al* (Curto *et al.*, 1998b)

4.3.2 Adding reactions of thiopurine metabolism to the model of purine metabolism

Reactions of thiopurine metabolism were added to the existing model of endogenous purine metabolism to produce a model shown in Figure 4.3, using the GMA type kinetic laws. The initial assumption was that all thiopurine reactions would be modified by endogenous metabolites and thiopurine metabolites and that all thiopurine metabolites behaved in the same way as their analogues. However this led to rate laws with many parameters that could not be defined with any confidence using data from the literature or experiments performed in this project. It was therefore decided that thiopurine substrates would be included in the rate law and that endogenous substrates will only act as modifiers in the same manner that they act on analogous endogenous purine reactions. Constants were unknown in the methylation reactions iTPMT, gTPMT and mpTPMT, and they were initially set to the default value 0.1. Thiopurine metabolites had an initial concentration of 0, except external MP to simulate drug being added to untreated cells.

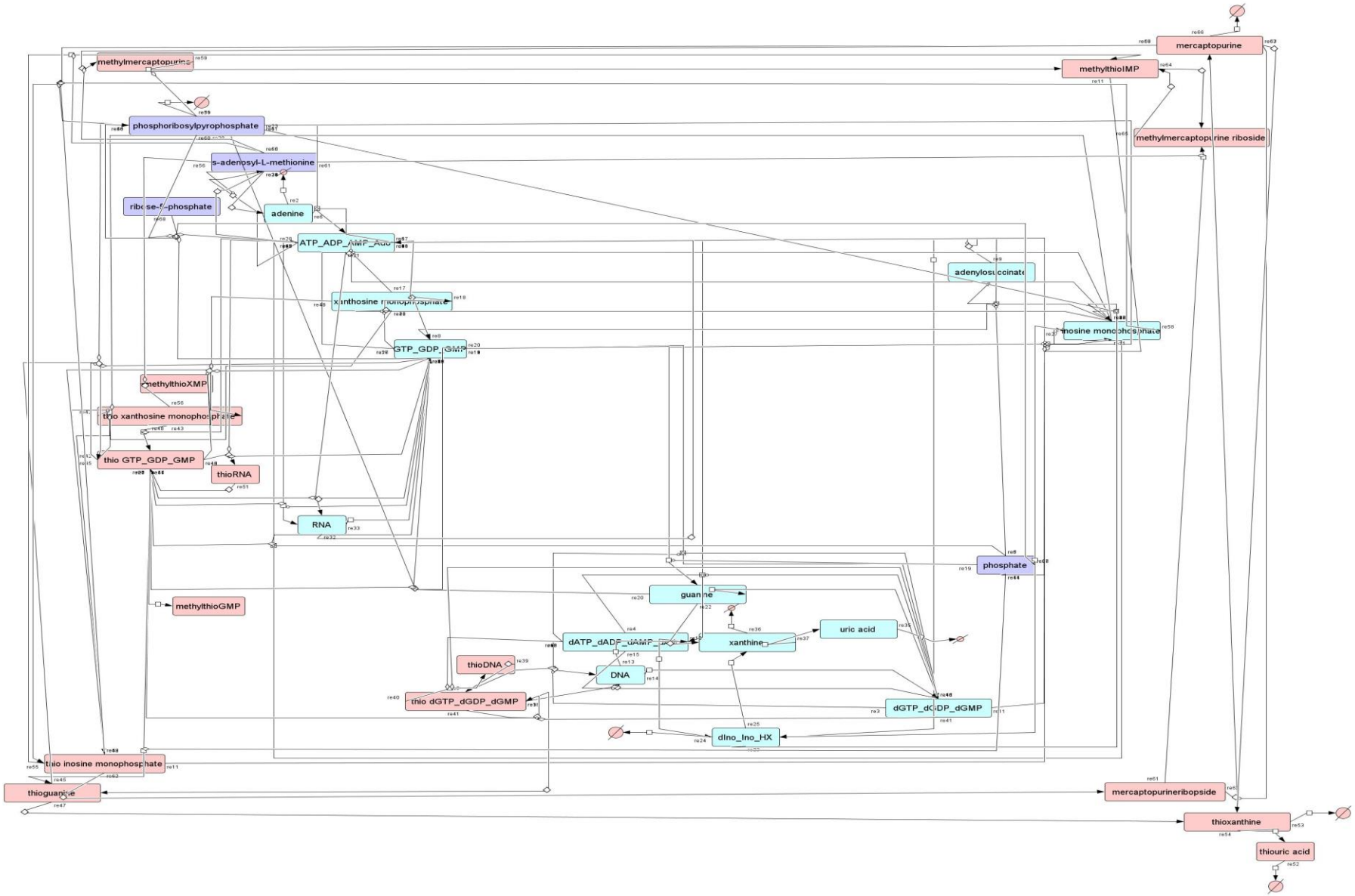

methylmercaptopurine
phosphoribosylpyrophosphate
s-adenosyl-L-methionine
adenine
ATP_ADP_AMP_Ade
xanthosine monophosphate
GTP_GDP_GMP
methylthioXMP
thio xanthosine monophosphate
thio GTP_GDP_GMP
thioRNA
RNA
methylthioGMP
thio inosine monophosphate
thioguanine
mercaptopurine
methylthioIMP
methylmercaptopurine riboside
adenylosuccinate
inosine monophosphate
phosphate
guanine
dATP_dADP_dAMP_dX
xanthine
uric acid
thioDNA
DNA
thio dGTP_dGDP_dGMP
dGTP_dGDP_dGMP
dIno_Ino_HX
mercaptopurineribopside
thioxanthine
thiouric acid

Figure 4.3 (Previous page) A graphical representation from CellDesigner of a version of a model combining thiopurine and endogenous purine metabolism with the purine metabolism following the Curto model. Endogenous purines shown in blue equivalent to the reactions shown in Figure 4.1, thiopurine metabolites; TIMP, TX, TUA, TXMP, TGMP, deoxyTGNs, TGNs in DNA and TGNS in RNA shown in red and thiopurine drugs MP and AZA shown in purple.

The GMA rate law (Figure 4.4) includes a rate constant, atgmps. This is multiplied by the concentration of the substrates or modifiers TXMP and ATP multiplied by the size of their compartment, in this case 1. Each concentration is raised to a power which indicates their effects, ftgmps7 and ftgmpsa. A positive number greater than 1 is an activator. A negative number or a fraction indicates an inhibitor (Voit and Savageau, 1987; Curto *et al.*, 1997). This type of rate law is not appropriate for species with a concentration of 0 as it produces the value infinity in the case of a negative power as shown in Table 4.3. This model was therefore rejected as GMA was unsuitable to model a time course following addition of MP to cells. This rejection was further supported by the fact that few parameters of the GMA rate laws could be established from the literature leaving many parameters unassigned making successful parameter estimation from data more difficult.

$$atgmps * (tXMP * default)\text{^}ftgmps7 * (ATP * default)\text{^}ftgmpsa/default$$

Figure 4.4 GMA type kinetic law for tGMPS from model of combined endogenous purine and thiopurine metabolism.

# Time	["thio GTP_GDP_GMP"]
0	0
0.72	-1.#IND
1.44	-1.#IND
2.16	-1.#IND
2.88	-1.#IND
3.6	-1.#IND

Table 4.3 An extract of data from a combined model of endogenous and thiopurine metabolism for combined TGN concentrations after time course simulation with thiopurine metabolites with an initial concentration of 0 using GMA kinetic laws an infinite value error message is displayed in column 2, this was true for all thiopurine metabolites

4.3.3 Variability of endogenous purines in cell culture samples as measured by LC-MS/MS

As described in Chapter 3 there was a great deal of variability in the levels of the endogenous purines measured by LC-MS/MS, ATP and GTP. This may be due to the fact that these measurements were made during method development before the importance of the temperature at which buffers were prepared was recognised, although they were variable even by comparison to thiopurine metabolites measured at the same time. Due to this variability the data concerning ATP and GTP would not be of sufficiently high quality to be used to estimate the inhibition constants needed to model the inhibition of the combination of reactions representing DNPS in the Curto model (Curto *et al.*, 1998b), by meTIMP. Therefore, the model finally built for this project was restricted to thiopurine metabolism. There was a great deal of variability in the amount of ATP detected by this LC-MS/MS method in MOLT-4 cells (Figure 4.5) with ATP levels normalised to protein concentration varying between samples grown under the same conditions. This may be due to the fact that this method is not optimised for separation of ATP from other chemicals with similar characteristics or because cells rapidly use ATP when stressed, as they may be during harvest. These data reinforced the fact that the model should deal only with thiopurines.

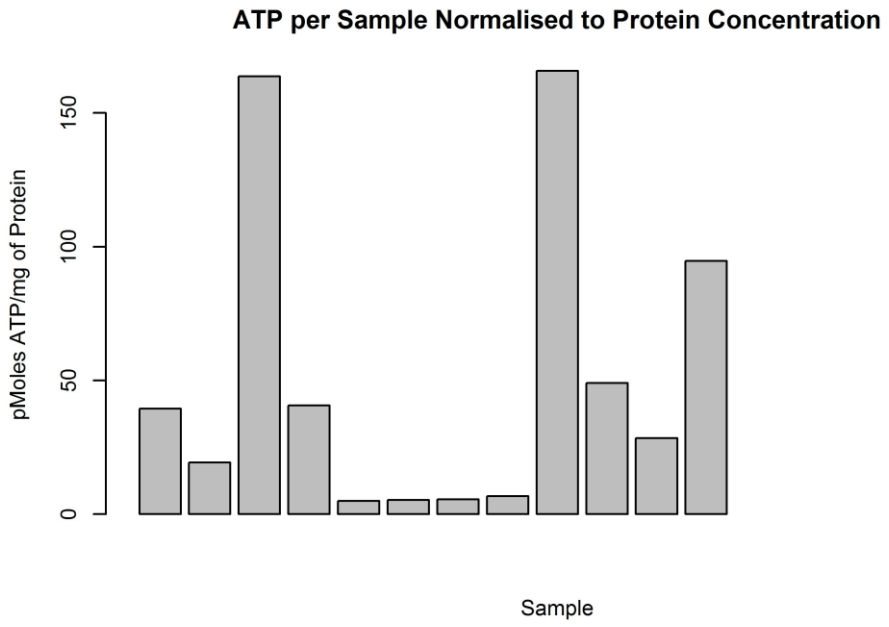


Figure 4.5 Bar chart showing ATP normalised to protein in cultured cells grown without MP.

4.4 Discussion

4.4.1 Comparison of the model as downloaded from BioModels database with the description of the model in Curto et al. (Curto et al., 1998b)

The version of the model of purine metabolism (Curto *et al.*, 1998b) downloaded from BioModels database (Chen *et al.*, 2010) (Figure 4.1) behaves as reported for the original version. It was able to replicate up to 100-fold variation in some reaction parameters and a 10-fold variation in the concentration of some species (Figure 4.2, Table 4.1 and Table 4.2). This also suggests that this model can replicate clinical effects of alterations in purine metabolism.

One difference between the model as published and the model as downloaded from BioModels database was that the model as published did not include the concentrations of species leaving the model, the products of excretion reactions or reactions which join other parts of cellular metabolism that use purines. Therefore in the downloaded version of the model these species are counted and constantly accumulate. This prevents a steady state analysis being run, which in turn prevented a full comparison with the model as previously described.

4.4.2 Suitability of power law kinetics

It has also been demonstrated that the GMA power law kinetic rate law is unsuitable for models in which the concentration of some species can become 0 (Table 4.3). This presents a problem as the model of thiopurine metabolism is intended to model a time course beginning with the addition of drug, when the metabolites of MP are at a concentration of 0. This type of model also requires many parameters for which data are lacking.

4.4.3 Measurements of endogenous purines

There were several important links between thiopurine metabolism and endogenous purine metabolism. MeTIMP inhibits DNPS; MTAP, an enzyme of adenosine salvage, alters the sensitivity of cell culture cells to thiopurines and alters the activity of TPMT. Also it is possible that there may be competition between thiopurines and endogenous purines for any enzymes that are at low concentrations. To use a combined model of purine and thiopurine metabolism, such as the one shown in Figure 4.3, to study these effects it would be necessary to estimate the kinetic constants representing these interactions and this would require experimental data about the effects of thiopurine treatment on

endogenous purine metabolism and vice versa to be collected. However the LC-MS/MS method was unable to reliably detect ATP (Figure 4.5), GTP or any other endogenous purine. Furthermore the MTAP reaction is not explicitly included in the Curto model (Curto *et al.*, 1998b) and therefore, without further data, it would be difficult to model the effects of MTAP deficiency on the concentrations of purines or thiopurines. As described above, detailed modelling of enzymatic reactions requires information about the concentrations of the enzymes and any co-factors, data that are not available in the literature. For these reasons a combined model of endogenous purine and thiopurine metabolism is unlikely to be successfully parameterised and validated at this stage.

4.5 Conclusions

The version of the model of purine metabolism developed by Curto *et al* (Curto *et al.*, 1998b) and downloaded from BioModels database (Chen *et al.*, 2010) was largely able to replicate the reported output with regards to several manipulations of both the initial values of species and of the rates of several reactions (Figure 4.2, Table 4.1 and Table 4.2). The rate law used in this model was unsuitable to model the metabolism of MP.

There were insufficient data available in the literature about the interactions of endogenous purine metabolism and thiopurine metabolism to be able to create and validate a combined model. The LC-MS/MS method developed during this project was not capable of producing data to inform this part of the model. For these two reasons a model of thiopurine metabolism alone was developed further, and this model is presented in Chapter 5.

Chapter 5. Building a model of thiopurine metabolism and using it to understand the mechanisms of the interaction between allopurinol and thiopurine metabolism

5.1 Introduction

5.1.1 Previous models of thiopurine metabolism

There have been two previous attempts at modelling different aspects of thiopurine metabolism and toxicity. One is a pharmacokinetic model of AZA metabolism and the other is a small scale dynamic model of the effects of MP on the viability of cells (Fransson *et al.*, 2006; Panetta *et al.*, 2006; Fransson *et al.*, 2007).

The model of the pharmacokinetics of thiopurines deals with the initial period after initiating AZA treatment. This model uses pharmacokinetic equations to represent the passage of the drug and metabolites through different tissues of the body and mass action kinetic equations to represent the action of metabolising enzymes on the thiopurines. The ultimate aim of this model was to predict the optimum dose needed to produce adequate levels of TGNs in a patient without producing too many methylated metabolites. However the model was unable to fit the data from the initial group of patients (Fransson *et al.*, 2006; Fransson *et al.*, 2007). The authors speculated that because there were only data available about two metabolites in one tissue this did not allow adequate fitting of a model which included reactions involving transport between several tissues and reactions of many other metabolites.

The other previous model of thiopurines was a model of the effects of TGN incorporation on cell viability (Panetta *et al.*, 2006). Data from three cell lines with different sensitivities to MP were fitted to the model and the estimated kinetic parameters for different reactions were compared. The cell culture data indicated that the difference in sensitivity to thiopurines between the cell lines was not due to their ability to incorporate TGNs into DNA. The model suggested that it was likely due to differences in their ability to progress through the cell cycle after incorporation of TGNs into DNA. This was confirmed by gene expression analysis showing altered levels of expression of genes known to be involved in control of the cell cycle (Panetta *et al.*, 2006).

5.1.2 XO Inhibitors

The XO inhibitor allopurinol is used clinically in combination with AZA to optimise the ratio of TGNs to methylated thiopurine metabolites (Sparrow *et al.*, 2005; Leung *et al.*, 2009; Brackett *et al.*, 2014). The mechanism of this optimisation is poorly understood, it would be expected that if XO inhibition, blocking excretion of MP via TX and TUA, was the only effect of allopurinol, then all metabolites would increase, thus maintaining the same ratio of TGNs to methylated thiopurine metabolites. In contrast, clinical data (Sparrow *et al.*, 2005; Leung *et al.*, 2009; Brackett *et al.*, 2014) indicate that with allopurinol, levels of methylated metabolites are reduced whilst TGNs increase. To explain this unexpected effect, it has been suggested that allopurinol might inhibit TPMT, possibly via TX (Blakera *et al.*, 2012); this has been shown to occur in RBC lysates but has not been demonstrated *in vivo* (Seinen *et al.*, 2013a). It has also been suggested that allopurinol may increase the activity of HGPRT although it has not been shown whether this is via an increase in HGPRT expression or via an activating interaction of allopurinol with the enzyme. A computational model of thiopurine metabolism will allow the consequences of various mode of allopurinol action to be assessed and facilitate the design of experimental tests to establish the mechanism of interaction of allopurinol with thiopurine drugs.

5.1.3 Aims

1. To develop a model able to fit previously available and newly generated data about thiopurine drug metabolism, specifically MP using cell culture samples.
2. To generate quantitative data from a time course of MP sensitive cells treated with MP to inform and validate a model of MP metabolism.
3. To expand the model to include potential interactions of allopurinol with MP drug metabolism, to attempt to explain the decreased sensitivity to MP of cells also treated with high concentrations of allopurinol and increased sensitivities to MP with cells treated with low concentrations of allopurinol and if possible the effect of allopurinol on methylated metabolite to TGN ratios seen in clinical data.

5.2 Methods

5.2.1 Growth and analysis of cell culture cells with MP

MOLT-4 cells were grown as described in 2.2.1.

Cells were seeded at $5x10^5$ cells/mL in 75 mL total initial volume for the MP-only time course and 80 mL total initial volume for allopurinol plus MP, 24 hrs before the time course experiment to recover from seeding and to reach $1x10^6$ cells/mL.

At the beginning of the experiment (t=0) a 6 mL aliquot of cells was removed, washed in PBS, split into two and snap frozen.

Drug was then added to cell culture dissolved in 0.1 M NaOH and medium to give a final concentration of 10 µM MP with 250 µM allopurinol or 1 mM allopurinol alone (from stock solution described in 2.2.3).

At 2, 4, 6, 8, 10 and 24 hours after addition of drug 6 mL aliquots of cells were removed, washed in PBS, split into two and snap frozen. When the experiment was repeated with allopurinol as well as MP technical replicates were omitted from LC-MS/MS analysis.

Cells were stored at -80°C until analysis by LC-MS/MS with results normalised to sample protein concentration. Linear regression was performed on the peak areas of the standards for each analyte and the sample values calculated using the intercept and gradient found for each analyte and the detected peak area. The concentration was then normalised to protein concentration in the cell lysate, as determined by Pierce BCA assay described in 3.2.4.

5.2.2 CoPaSi 4.6.32

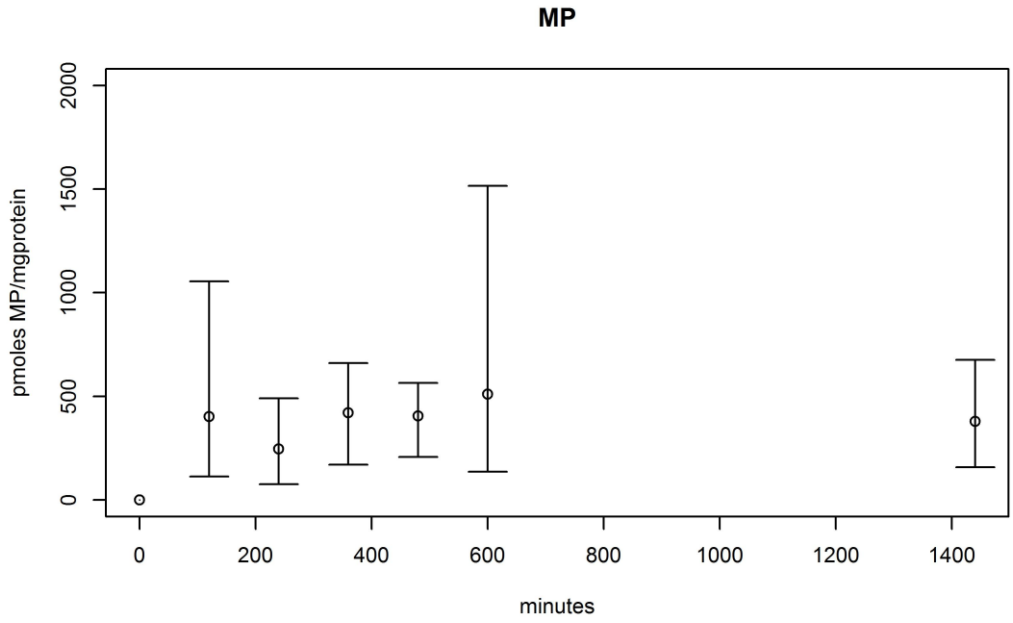
A model of MP metabolism was developed in CoPaSi (Hoops *et al.*, 2006) a programme for building and editing biological models described in section 4.2.4.

5.3 Results

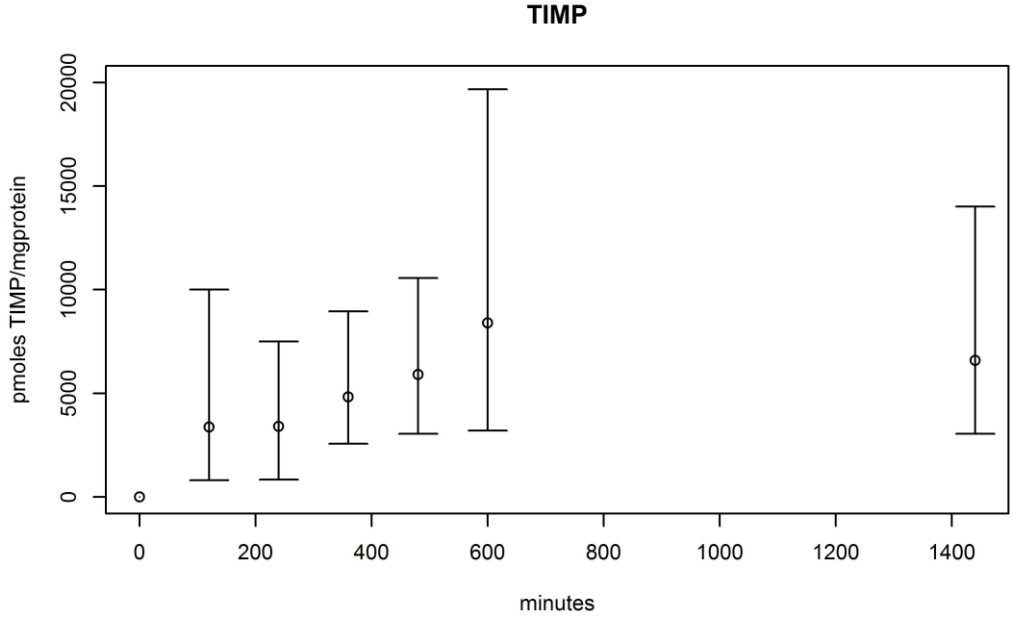
5.3.1 Cell culture data with MP alone

As shown in Figure 5.1 after treatment with 10 μM MP alone concentrations of meTIMP,DP and TP, meTGMP, DP and TP and TGMP, DP and TP increased throughout the 24 hours of the time course whilst the TIMP concentration increased initially before reaching a plateau or beginning to decrease at 10 h. MP increased initially before reaching a plateau after approximately 2 h.

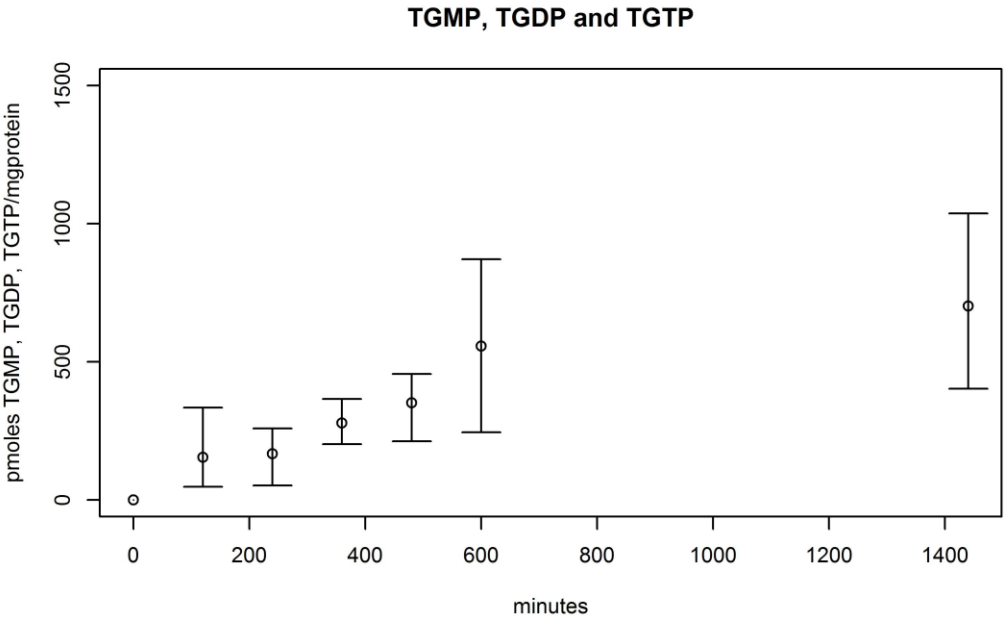
Variation was generally greater between biological replicates than between technical replicates (Table 5.1). Metabolites detected at higher levels such as TIMP were less variable than those nearer their limit of detection, for example meTGMP,DP,TP. Technical replicates were only occasionally more than 50% and were mainly less than 20% whereas biological replicates often varied by more than 50%.


MP
pmoles MP/mgprotein
0
500
1000
1500
2000
0
200
400
600
800
1000
1200
1400
minutes

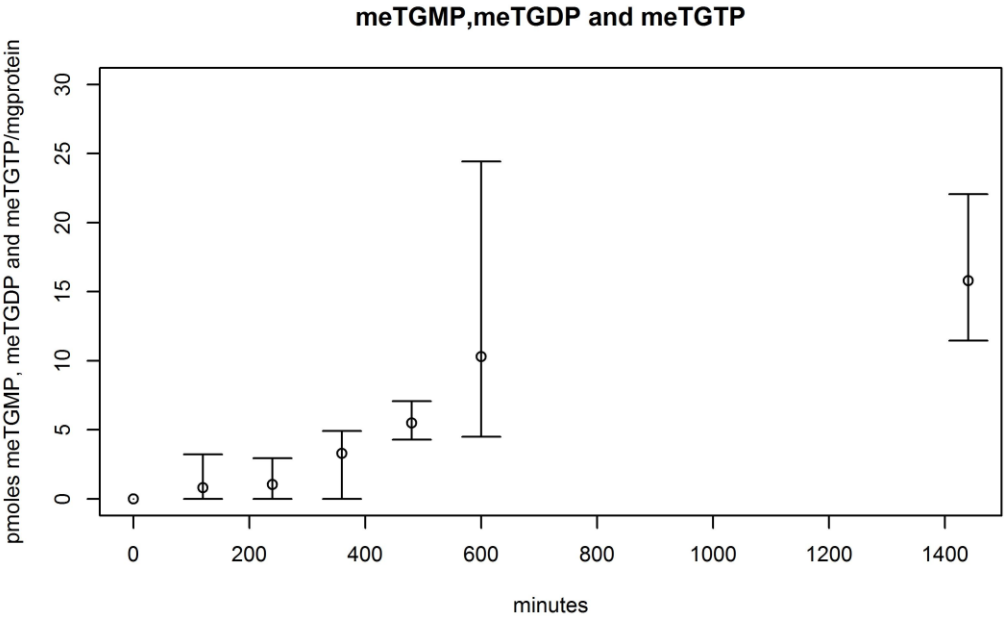
a


TIMP
pmoles TIMP/mgprotein
0
5000
10000
15000
20000
0
200
400
600
800
1000
1200
1400
minutes

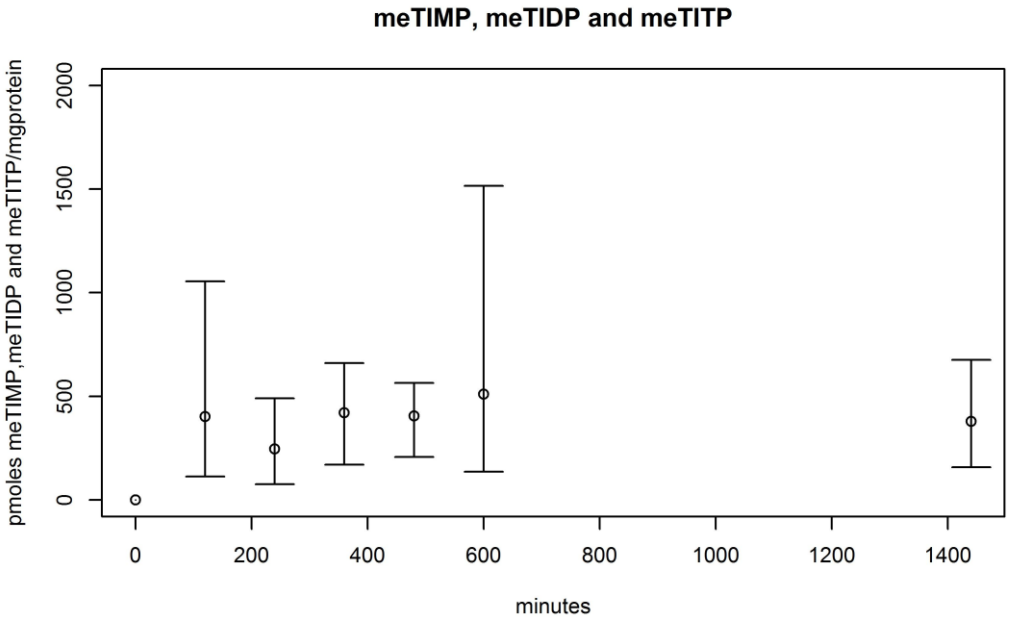
b


TGMP, TGDP and TGTP
pmoles TGMP, TGDP, TGTP/mgprotein
0
500
1000
1500
0
200
400
600
800
1000
1200
1400
minutes

c


meTGMP,meTGDP and meTGTP
pmoles meTGMP, meTGDP and meTGTP/mgprotein
0
5
10
15
20
25
30
0
200
400
600
800
1000
1200
1400
minutes

d



e

Figure 5.1 a-e Graphs showing the mean (points [n=3 biological replicates]) and 1 SD of the concentrations of metabolites of MP normalised to protein concentration from MOLT-4 cells cultured with 10 µM MP **a** MP, **b** TIMP **c** TGMP, TGDP and TGTP summed, **d** meTGMP, meTGDP and meTGTP summed, **e** meTIMP, meTIDP and meTITP summed.

Analyte	Range of % co efficient of variation of biological replicates	Range of % co efficient of variation of technical replicates
TIMP	55-109	1-73
MP	34-101	12-118
TGMP,DP,TP	23-73	1-88
meTIMP,DP,TP	28-58	4-88
meTGMP,DP,TP	18-167	1-141

Table 5.1 The percentage co-efficient of variation between different repeats of MOLT-4 cells grown with MP.

5.3.2 Initial model development in CoPaSi

This model was developed in CoPaSi (Hoops *et al.*, 2006) with all reactions and species defined *ab initio*. The reactions included were those described in Figure1.2 that relate to MP. Initially the reduction of TGMP by GMP reductase was also included however evidence suggests that TGMP is not a substrate for this enzyme (Spector *et al.*, 1979). The model assumed Michaelis–Menten kinetics (Michaelis and Menten, 1913; Johnson and Goody, 2011) for most reactions. Michaelis-Menton kinetics is an approximation of enzyme substrate binding and product release which assumes an excess of enzyme relative to the concentration of substrate. Michaelis-Menten kinetics does not take account of any inhibition or activation by substrates, products, co-factors or competitive or allosteric modifiers. This reduces the number of parameters in the model compared to rate laws used in the earlier model described in Chapter 4 and as there were limited data available about the rate laws appropriate to thiopurine metabolism, this was an appropriate simplification.

Km values for the various enzymes were obtained from the literature; where a Km for a thiopurine metabolite was unavailable, it was assumed to be three times larger than that of the endogenous metabolite (affinity reduced to one third the endogenous affinity). This was approximately the case for XO in relation to TX in a study in which both values were reported (Kudo *et al.*, 2010). Initial Km values are listed in Table 5.2.

The only exception to this was incorporation of thioguanosine into DNA as this represents several steps the intermediates of which were not measured. This was modelled using a mass-action rate law, assuming the reactions to be dependent on the availability of TGNs and nothing else.

Values were transformed to internal cell concentration in µM based on a cell volume of 2 pL (2e-12 L) and 100 µg protein per cell (Rodríguez-Caso *et al.*, 2006) except for MP_out which was placed in a separate compartment of volume 0.07 L.

All species values except MP_out, the concentration of MP present in the medium were initially defined as 0 and MP_out was initially defined as 10 µM, as this was the concentration of MP incubated with cells.

Parameter	Initial Value (If derived from literature)	Value estimated from mean data	Value estimated from separated biological replicates
K1 DNA incorporation (1/min)		0.04	0.0281434
Km GMPS (µ mol/L)	12.00(3.6 X 3) (Spector, 1975)	68558e+13	1.80643e+12
Vmax GMPS(µ mol/(L*min)		3.53686e+11	1.06985e+10
Km HGPRT I (µ mol/L)	18.80 (Lennard *et al.*, 1993)	548.22	635.22
Vmax HGPRT I (µ mol/(L*min)		0.09	0.09
Km HGPRT G (µ mol/L)	18.80	18.80	18.80
Vmax HGPRT G(µ mol/(L*min)		43.25	39.29
Km IMPDH(µ mol/L)	135.00 (45 X 3) (Yamada *et al.*, 1990)	4815.39	28977.90
Vmax IMPDH(µ mol/(L*min)		20.00	99.98
Km MP uptake(µ mol/L)	14.21(Katsuhito *et al.*, 2007)	0.12	1.06
Vmax MP uptake(µ mol/(L*min)		1.79258e-11	1.54676e-11
Km TPMTMP(µ mol/L)	10.60 (Krynetski *et al.*, 1995)	10.60	10.60

Vmax TPMTMP(µ mol/(L*min)		9.77	8.019
Km TPMPTtGMP (µ mol/L)	27.10 (Krynetski *et al.*, 1995)	27.10	27.10
Vmax TPMTtGMP (µ mol/(L*min)		0.0014	0.0015
Km TPMTtIMPuptake(µ mol/L)	25.70 (Krynetski *et al.*, 1995)	25.70	25.70
Vmax TPMTtIMP (µ mol/(L*min)		0.086	0.089
Km XOMP (µ mol/L)	4.97 (assumed equal to XOtX)	2.50	2.50
Vmax XOMP (µ mol/(L*min)		1.14	1.14
Km XOtX (µ mol/L)	4.97 (Kudo *et al.*, 2010)	4.97	4.97
Vmax XOtX(µ mol/(L*min)		0.37	0.37

Table 5.2 (previous page) Values of parameters as determined from the literature (column 2), by parameter estimation using CoPaSi and the mean of 3 biological and 2 technical replicates (6 total) (column 3), and each biological replicate as a separate experiment with 2 repeats (column 4).

V max values were determined using the parameter estimation tool in CoPaSi. The initial values were determined by approximately replicating the cell culture data by manipulating the V max values manually beginning at 0.1. This was followed by using the Parameter Estimation facility in CoPaSi. The random search, evolutionary programming, particle swarm and Hooke and Jeeves algorithms from CoPaSi were used and the model was updated after each run. The Hooke and Jeeves algorithm is a direct search algorithm. It does not rely on differentiation, but instead on an iterative calculation of the value of the function of the differences between data and the model. Evolutionary programming creates a population of possible parameter sets; each parameter is equivalent to a gene, these are then each compared to several others allowing individuals to be ranked and the worst are eliminated. Small random changes, or mutations, are made and the new population is then re-ranked; after a number of iterations the best genome is selected. The random search algorithm generates random parameter sets and compares them to the desired outcome; given unlimited iterations this algorithm will produce an ideal solution. The particle swarm method begins with a swarm of possible parameter sets or particles these are altered to change the position of the particles in the parameter space; these solutions are then compared and the best position is updated, eventually this may lead to the optimal parameter set being found, although a local optimum may be found instead. Runs were performed iteratively until no further reduction in the sum of squares was seen; if one of the parameters had reached its boundary, the range for that parameter was extended and the estimation run again. As this strategy did not result in a good fit alone, Km values for GMPS, IMPDH, MP uptake, XOMP and HGPRT were also allowed to vary; each was added in turn to the list of parameters to be estimated when no improvement in model fit, compared to data from cultured cells, was seen. These parameters were chosen to vary for a variety of reasons; GMPS and IMPDH were allowed to vary first as the Km values reported for these were for endogenous purines, MP uptake was based on the Km of only one transporter not all possible transporters, XOMP was based on data for XO with TX not MP, and HGPRT was based on data from RBCs not MOLT-4s.

Data used for parameter estimation with CoPaSi were time course data from cells grown with 10 µM MP for 24 hours and then analysed using LC-MS/MS as

described above. Constraints were also placed upon the parameter estimation, an option available in the CoPaSi parameter estimation tool. These were that TGMP<TXMP<TIMP as this had previously been described under the similar conditions by a group able to measure TXMP (Zimm *et al.*, 1985). Also, models that did not fulfil the condition that at early time points TIMP should be substantially higher concentration than TUA (Kalra *et al.*, 2007) were rejected after a time course simulation had been run. Parameter estimations were run with the data either entered as three separate experiments, each with two repeats representing technical replicates, or with a mean of all replicates.

Time course data were produced using the deterministic (LSODA) simulator in CoPaSi. Time course data were obtained for the model with 10 µM MP. An extra parameter was added to the model to simulate changes in TPMT activity. Time course simulations were run to compare this model against data from two published papers describing knock down or increased TPMT activity (Coulthard *et al.*, 2002; Misdaq *et al.*, 2012). Time courses were also simulated with 10 µM MP and varying levels of TPMT activity.

A graphical representation of the model is shown in Figure 5.2 and SBML of the model is in appendix a.

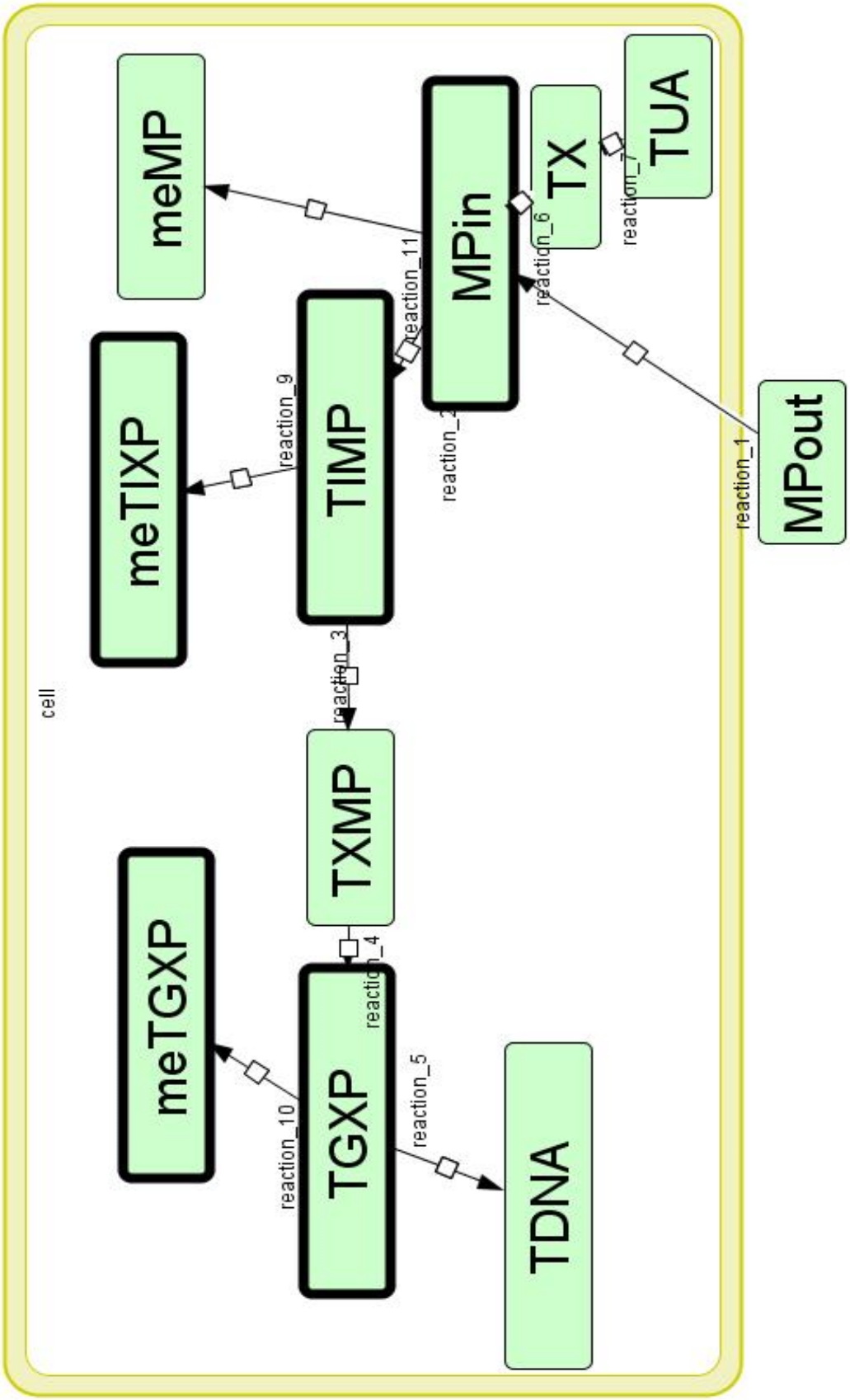


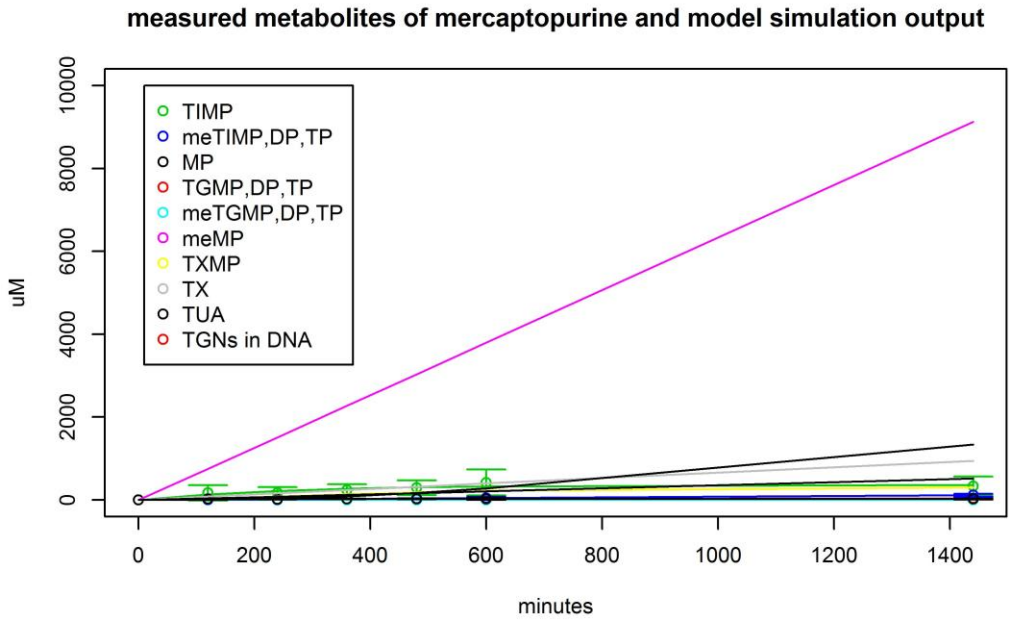
Figure 5.2 Graphical representation of the initial model of MP metabolism without allopurinol as produced in Cell Designer from SBML imported from CoPaSi. Thiopurine metabolites or groups of metabolites measured by LC-MS/MS used in parameter estimation are highlighted by bold edging.

5.3.3 Parameter Estimation in CoPaSi of the model of MP metabolism

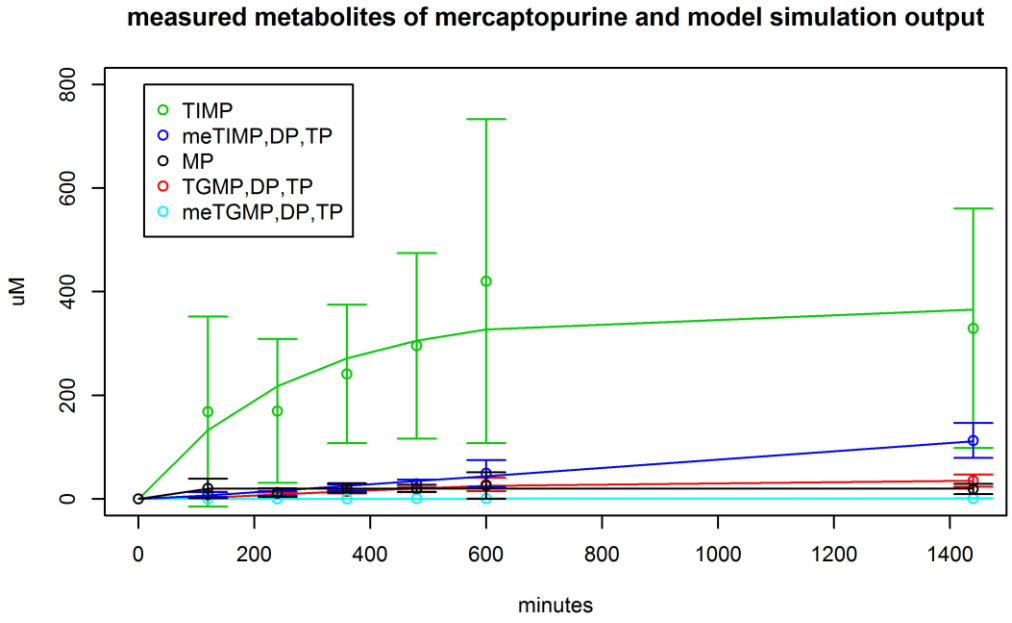
The SBML of this model is included in appendix a. Parameters were estimated in CoPaSi using data collected in two ways. Firstly, with the mean of all six replicates defined as one experiment CoPaSi (final parameters shown in Table 5.2 column 3). Secondly with the three biological replicates defined as three experiments with two replicates each in CoPaSi (final parameters shown in Table 5.2 column 4). Most parameters values were similar for the two methods of parameter estimation. However, GMPS Km and Vmax, IMPDH Km and 6-MP uptake Km all varied by 10 fold or more. The two variants of the model (informed by the two methods of parameter estimation) produced similar time course results (Figure 5.3 a and b compared to c and d). This similarity suggests that the most variable parameters are insensitive to change.

The model output and cell culture data are compared in Figure 5.3 b and d. The value reached for sum of squares of the parameters estimated from the mean data was 24.47.The value for sum of squares with the data separated by biological replicate was 1619.00. This is a much poorer fit probably due to the fact that the data loaded to CoPaSi was much more variable.

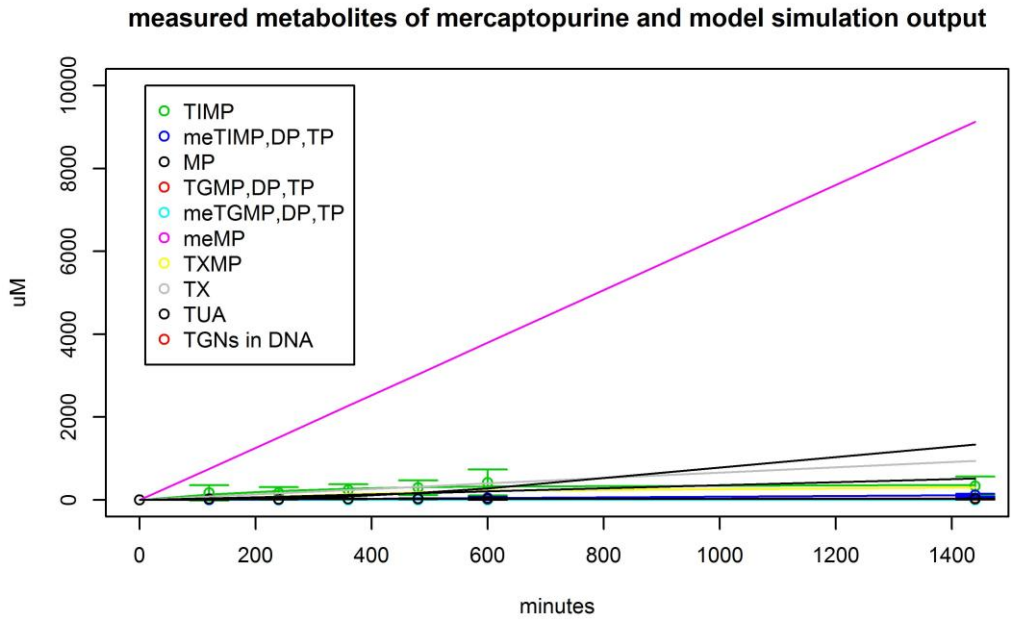
Due to the similarities of the model variants in terms of time course output and parameter values, and the better progress of fit value of the model variant estimated using the mean, all further investigations only used the model as parameterised using mean data.


measured metabolites of mercaptopurine and model simulation output
TIMP
meTIMP,DP,TP
MP
TGMP,DP,TP
meTGMP,DP,TP
meMP
TXMP
TX
TUA
TGNs in DNA
uM
0
2000
4000
6000
8000
10000
0
200
400
600
800
1000
1200
1400
minutes

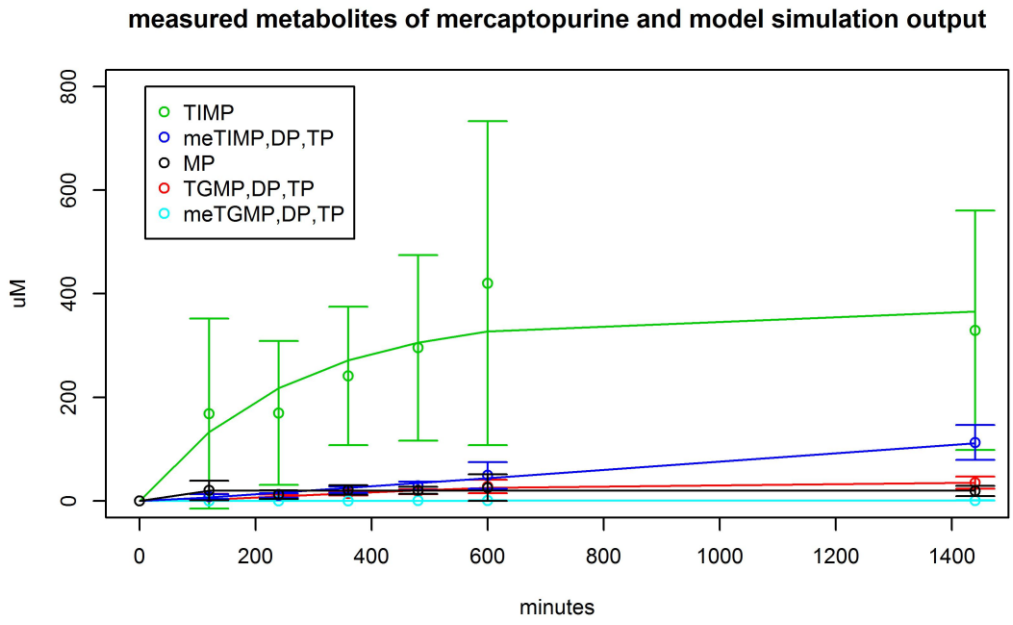
a


measured metabolites of mercaptopurine and model simulation output
TIMP
meTIMP,DP,TP
MP
TGMP,DP,TP
meTGMP,DP,TP
uM
0
200
400
600
800
0
200
400
600
800
1000
1200
1400
minutes

b


measured metabolites of mercaptopurine and model simulation output
TIMP
meTIMP,DP,TP
MP
TGMP,DP,TP
meTGMP,DP,TP
meMP
TXMP
TX
TUA
TGNs in DNA
uM
0
2000
4000
6000
8000
10000
0
200
400
600
800
1000
1200
1400
minutes

c


measured metabolites of mercaptopurine and model simulation output
TIMP
meTIMP,DP,TP
MP
TGMP,DP,TP
meTGMP,DP,TP
uM
0
200
400
600
800
0
200
400
600
800
1000
1200
1400
minutes

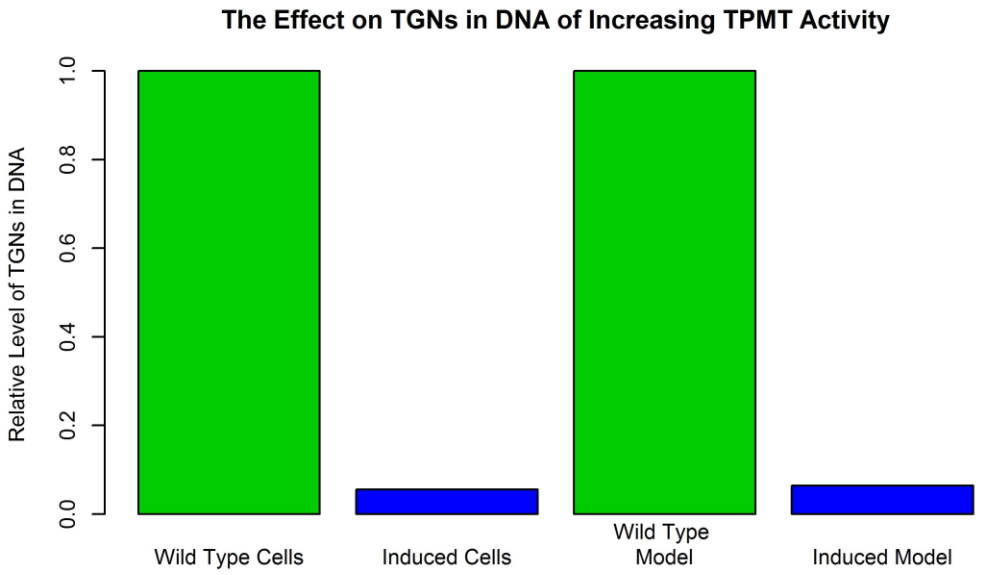
d

Figure 5.3 a-d (previous 2 pages) Graphs showing metabolite concentration data from MOLT-4 cells grown with 10 µM MP adjusted to µM inside cells mean as points with bars showing 1 SD (n= 3) and lines showing the output of simulations of the computational model of MP metabolism. **Green** TIMP, **dark blue** meTIMP,meTIDP and meTITP summed, **black** MP, **red** TGMP, TGDP and TGTP summed and **pale blue** meTGMP, meTGDP and meTGTP, **pink** meMP (model), **yellow** TXMP (model), **grey** TX (model), **black** TUA (model) and **red** TGNs in DNA (model). **a** Model output from model calibrated using mean data showing only those metabolites measured in cell culture, **b** Model output from model calibrated using mean data showing all metabolites included in the model, **c** Model output from model calibrated using repeated data as separate experiments showing all metabolites, **d** Model output from model calibrated using repeated data as separate experiments showing only those metabolites measured in cell culture. All units µM and minutes.

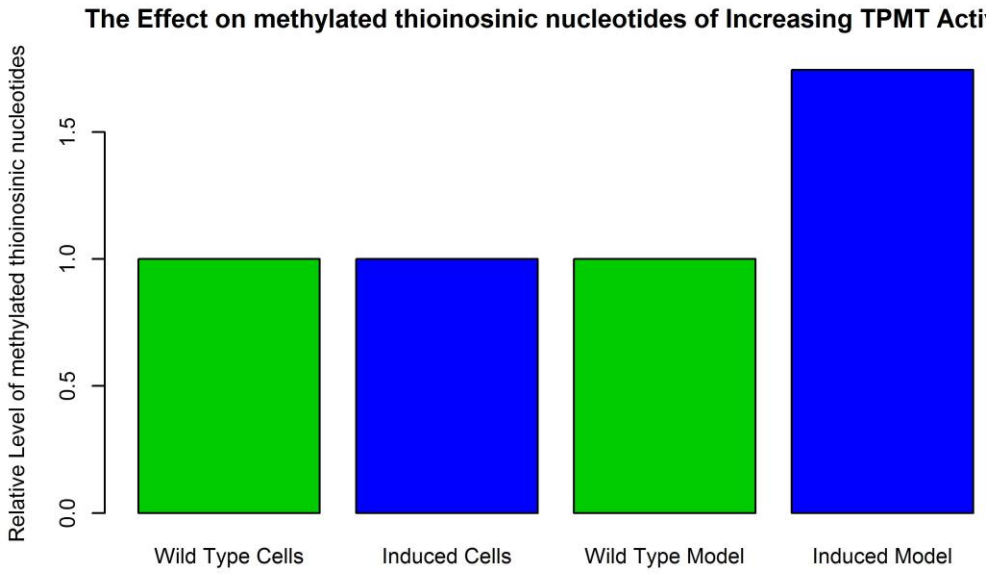
5.3.4 Response of the model of MP metabolism to variation in TPMT activity

Coulthard *et al.* (2002) describe the effect of a 3.8 fold increase in TPMT activity on the levels of methylated thiopurine metabolites and TGNs in DNA after incubation with equitoxic doses of MP (1.8 µM for cells induced to increase TPMT activity, 8 µM for normal cells). The paper showed that the level of meTIMP was increased but not significantly in cells with induced TPMT compared to uninduced cells treated with equitoxic doses of MP. This suggests that the effect of TPMT activity in the model may be too great with respect to meTIMP as the increase in the model under these circumstances is 68%, shown in (Figure 5.4b). The decrease in TGNs in DNA reported by Coulthard *et al* (Coulthard *et al.*, 2002) and that in the model are more similar in magnitude (Figure 5.4a).

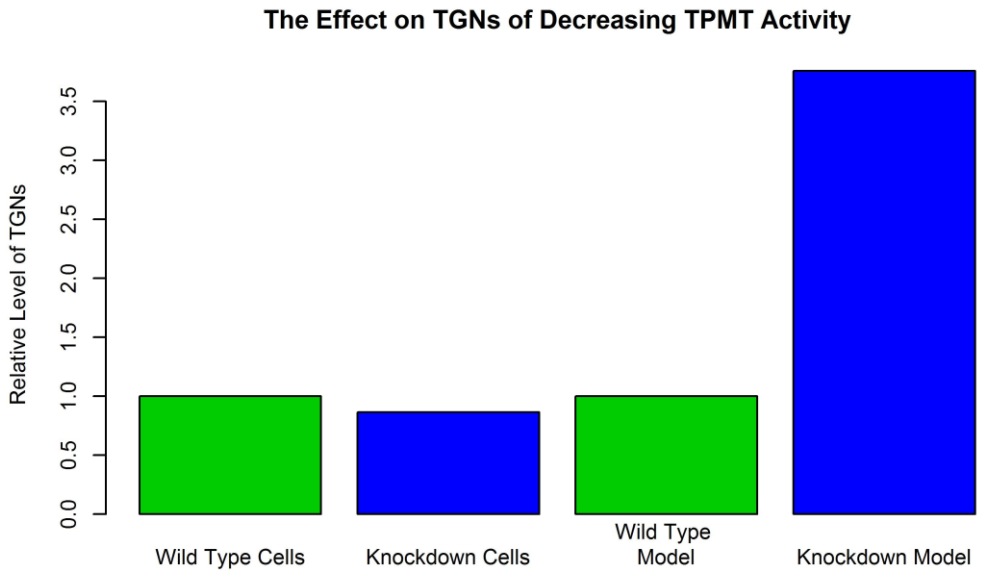
Misdaq *et al* (2012) describe the effects of reducing TPMT activity from 6.1 U/mg protein to 1.7 U/mg protein. This paper describes the effects of incubating TPMT knockdown and wild type Jurkat cells with 4.6 and 4.7 µM MP on TGNs and meMP. TGNs decreased, although not significantly, in the cells however, in the model they increase by 279% (Figure 5.4c). meMP decreased by 95% in Jurkat cells with knocked down TPMT compared to 62% in the same experiment in the model (Figure 5.4d). This suggests that with respect to the effect on TPMT inhibition the model is able to replicate data qualitatively from another cell line for meMP but not TGN concentration.


The Effect on TGNs in DNA of Increasing TPMT Activity
Relative Level of TGNs in DNA
0.0
0.2
0.4
0.6
0.8
1.0
Wild Type Cells
Induced Cells
Wild Type Model
Induced Model

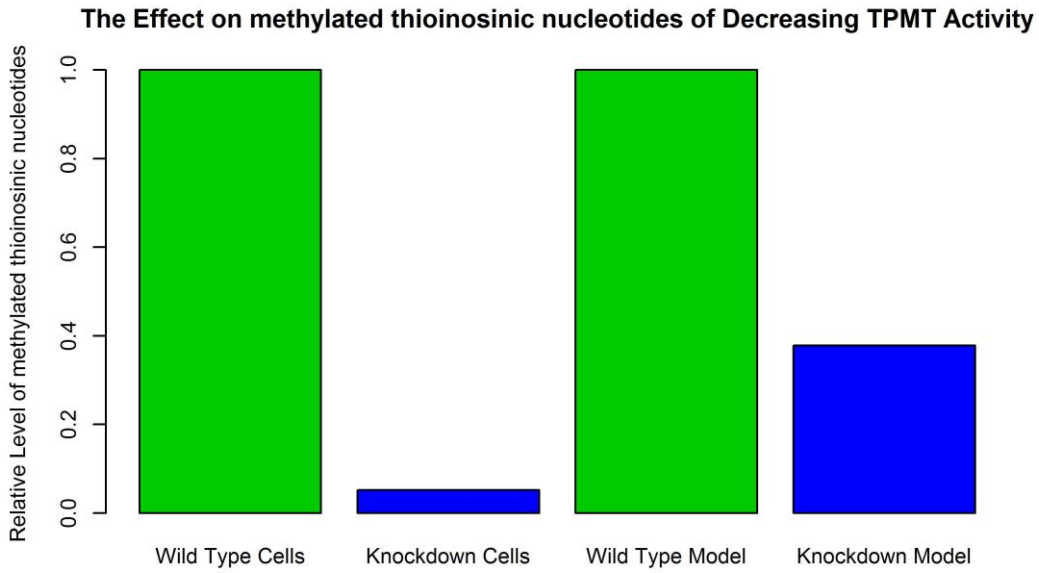
a


The Effect on methylated thioinosinic nucleotides of Increasing TPMT Activity
Relative Level of methylated thioinosinic nucleotides
0.0
0.5
1.0
1.5
Wild Type Cells
Induced Cells
Wild Type Model
Induced Model

b



c



d

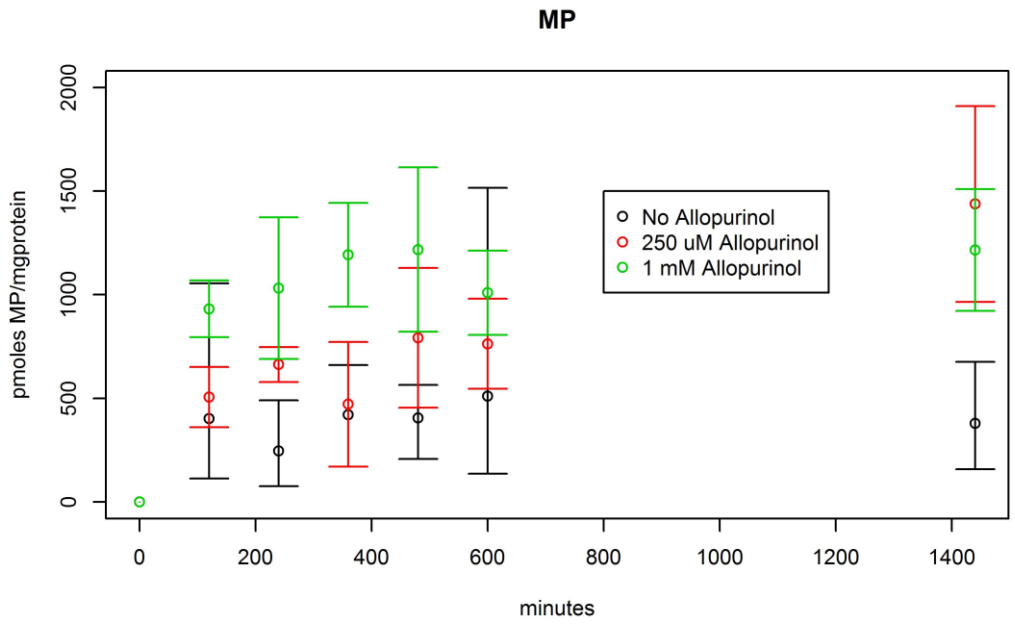
Figure 5.4 a-d (previous 2 pages) Showing existing data from papers compared to time course simulations from the model of thiopurine metabolism calibrated in CoPaSi. Data expressed as relative values to those with normal TPMT activity effects of **a** increasing TPMT activity on TGNs in DNA **b** increasing TPMT activity on methylated thioinosinic nucleotides **c** decreasing TPMT activity on TGNs **d** decreasing TPMT activity on methylated thioinosinic nucleotides.

5.3.5 Culture of cells with allopurinol and MP

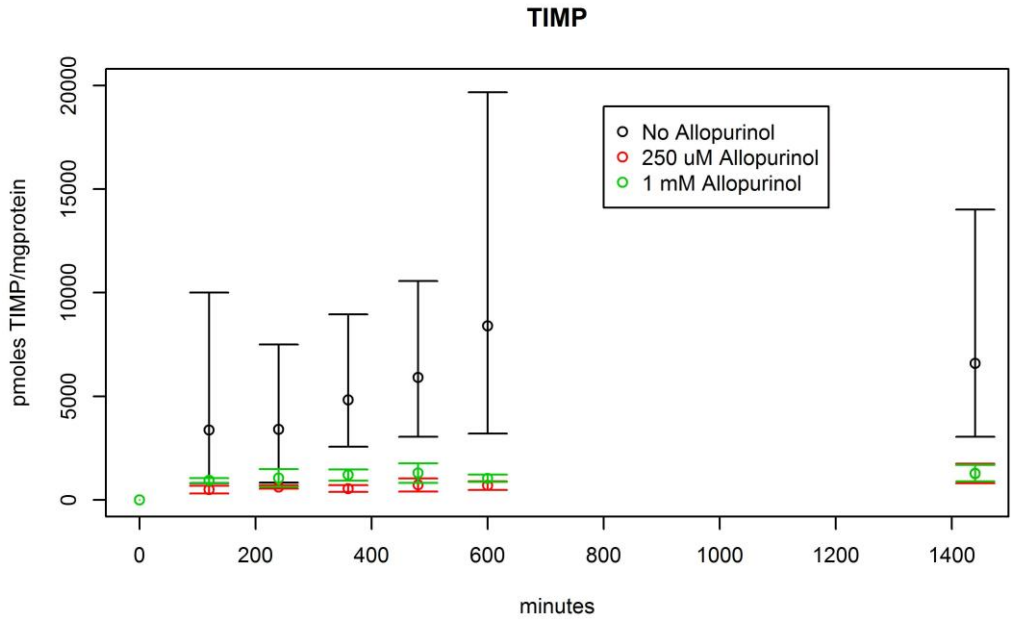
In the presence of either 1 mM or 250 µM allopurinol with 10 µM MP there was a lower concentration of all metabolites measured (Figure 5.5b-e), except for MP (Figure 5.5a), than there was when cells were grown with 10 µM MP alone. There was no reduction in the ratio of meTIMP, meTIDP and meTITP to TGMP, TGDP and TGTP at time points up to 24 hours.

5.3.6 Expansion of the model to include interactions between allopurinol and MP

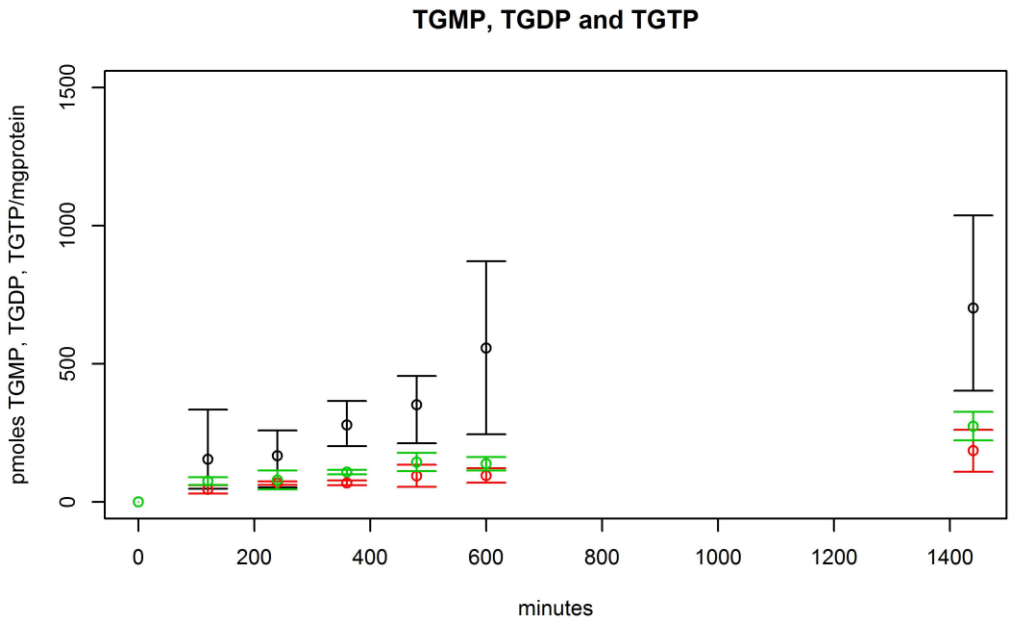
This model was adapted to represent possible effects of allopurinol on MP metabolism as shown in Table 5.3 and Figure 5.6. Allopurinol was assumed to act as a competitive inhibitor of XO. It was proposed that allopurinol was also a competitive inhibitor of HGPRT and, or MP uptake, as both it and MP are analogues of HX and the data from cells grown with allopurinol showed a reduction in the concentrations of MP metabolites. HGPRT activity has been shown to be induced by use of AZA combined with MP; however this may be due to induction of HGPRT expression after competition for the enzyme. It was also proposed that TPMT may be inhibited by TX which may increase after allopurinol treatment; this follows mechanistic reports in the literature. Each interaction was modelled using the competitive inhibition rate law pre-defined in CoPaSi; 8 versions of the model representing different combinations of these reaction were created (Figure 5.6 and Table 5.3).


MP
pmoles MP/mgprotein
0
500
1000
1500
2000
0
200
400
600
800
1000
1200
1400
minutes
No Allopurinol
250 uM Allopurinol
1 mM Allopurinol

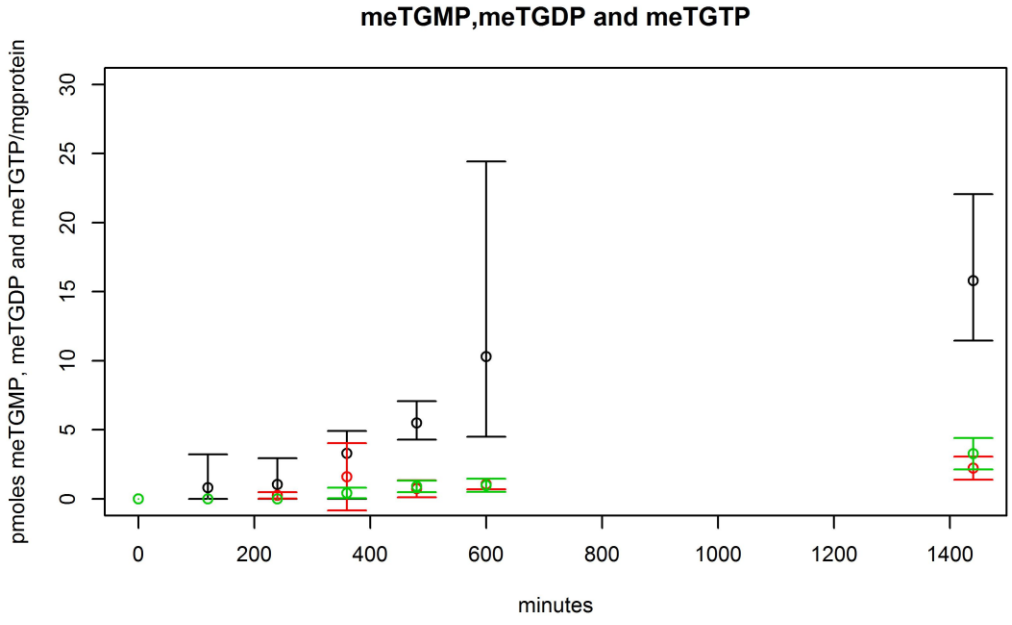
a


TIMP
pmoles TIMP/mgprotein
0
5000
10000
15000
20000
0
200
400
600
800
1000
1200
1400
minutes
No Allopurinol
250 uM Allopurinol
1 mM Allopurinol

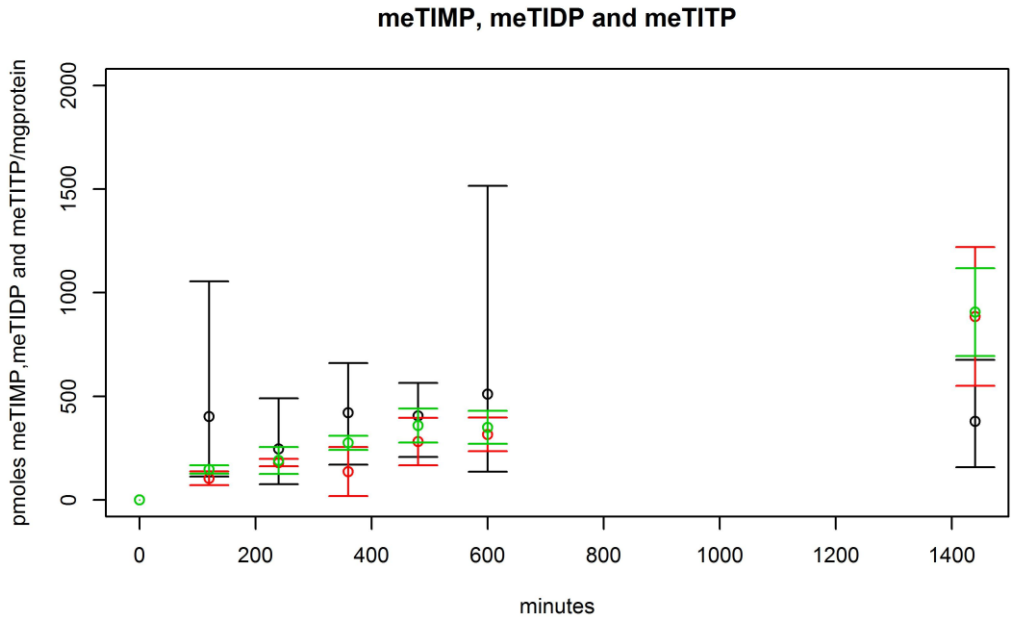
b


TGMP, TGDP and TGTP
pmoles TGMP, TGDP, TGTP/mgprotein
0
500
1000
1500
0
200
400
600
800
1000
1200
1400
minutes

c


meTGMP,meTGDP and meTGTP
pmoles meTGMP, meTGDP and meTGTP/mgprotein
0
5
10
15
20
25
30
0
200
400
600
800
1000
1200
1400
minutes

d



e

Figure 5.5 a-e Graphs showing the mean (points [n=3 biological replicates]) and 1 SD of the concentrations of metabolites of MP normalised to protein concentration from MOLT-4 cells cultured with 10 µM MP **a** MP, **b** TIMP **c** TGMP, TGDP and TGTP summed, **d** meTGMP, meTGDP and meTGTP summed, **e** meTIMP, meTIDP and meTITP summed.

Model/Affected Reaction	XO	TPMT (by TX)	HGPRT	MP Uptake
A	X	X	X	
B	X	X		X
C	X		X	X
D	X	X		
E	X		X	
F	X			X
G	X			
H	X	X	X	X

Table 5.3 Showing the proposed reactions of allopurinol on MP metabolism, X shows an inhibition included in the relevant version of the model.

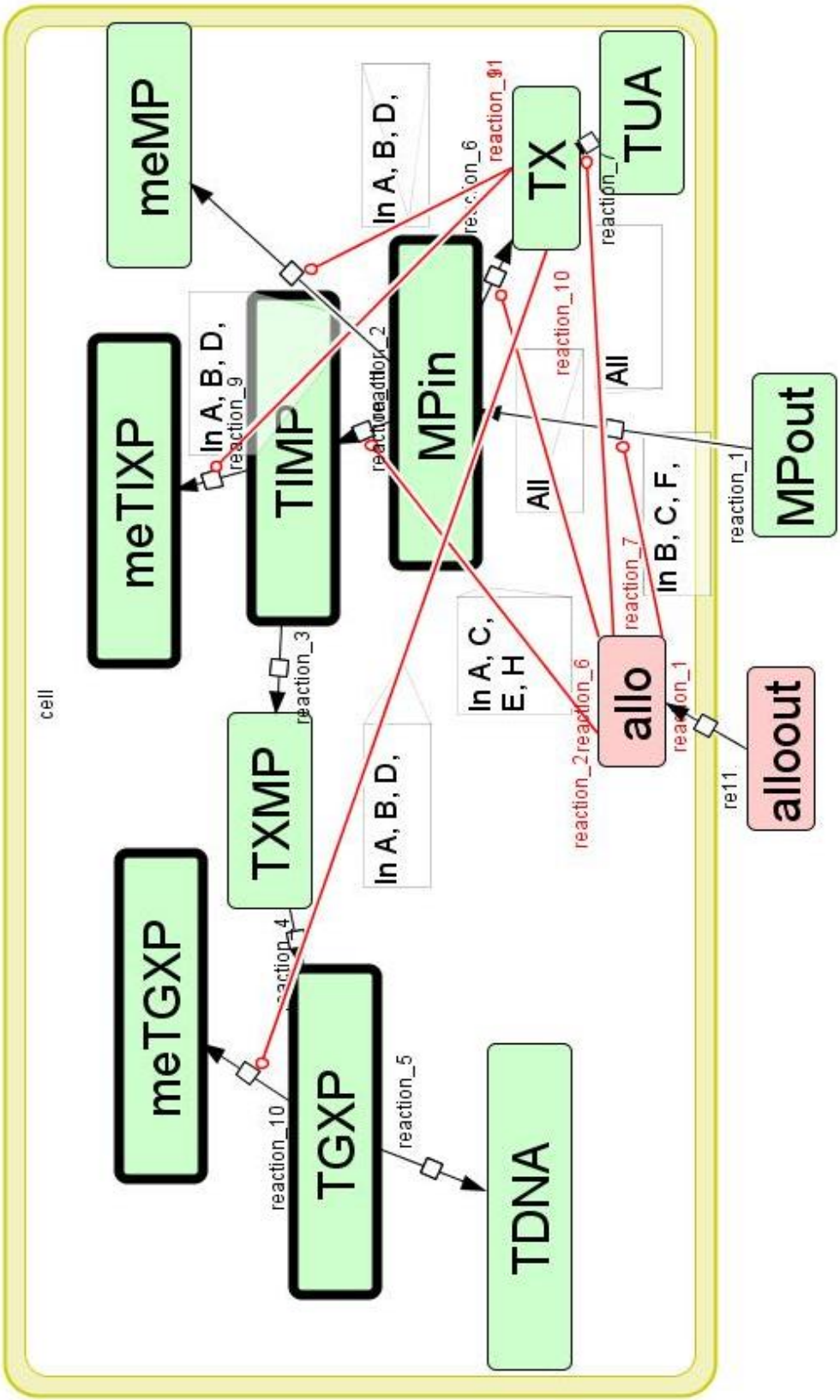


Figure 5.6 Graphical representation of the model variants showing the proposed effects of allopurinol on MP metabolism in red, which combinations of the interactions shown in red is included in each model variant is described in Table 5.3.

5.3.7 Parameter estimation in model variants with and without allopurinol

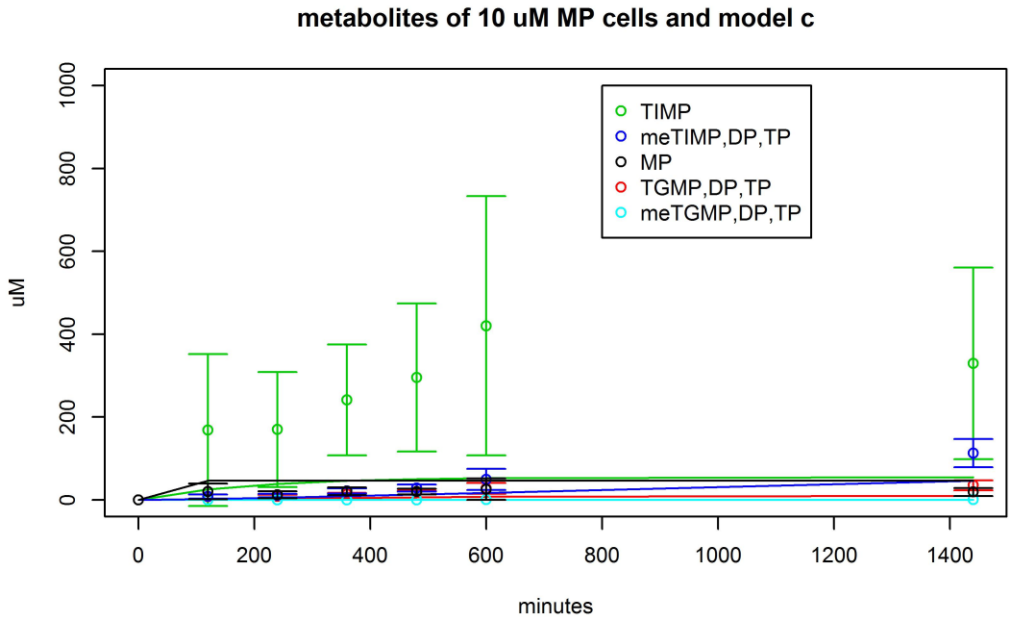
Parameter estimation, beginning with the model as developed for MP alone was performed as above, including the Ki's for each of the proposed reactions and the Km and Vmax of allopurinol uptake. The data used were from cell culture cells grown with 10 μM MP with either 1 mM allopurinol or 250 μM allopurinol. Parameter estimations were carried out by fitting the mean of three biological replicates for each of three treatments (0 allopurinol plus MP, 1 mM allopurinol plus MP and 250 μM allopurinol plus MP).

All versions of the model were then simulated with initial concentrations of 10 μM MP and either 0, 1 mM, 250 μM or 100 μM allopurinol.

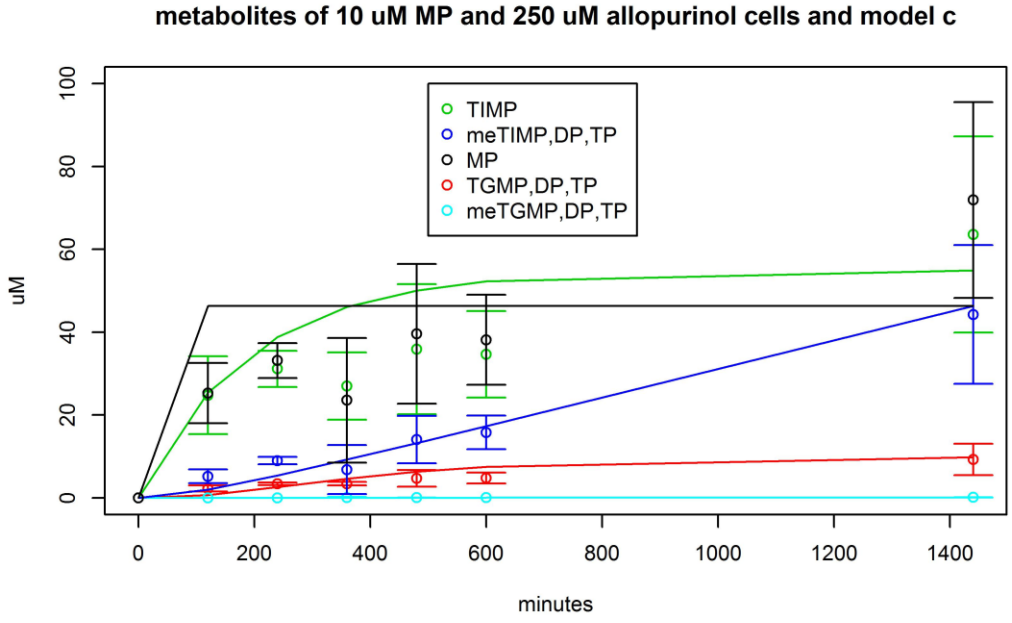
All 8 versions of the model, with variants on the possible interactions of allopurinol with MP metabolism specified in Table 5.3, were unable to simultaneously fit the three data sets, 10 μM MP alone and in combination with either 250 μM allopurinol or 1 mM allopurinol. The minimum value of sum of squares reached were; A 105, B 118, C 105, D 107, E 109, F 106, G 109, and H 108. These values are all similar but A (allopurinol inhibiting XO and HGPRT and TX inhibiting TPMT), and C (Figure 5.7) (allopurinol inhibiting XO, HGPRT and MP uptake) had slightly better fits than the rest.

All of the models had problems fitting the 10 μM MP with 1 mM allopurinol with respect to the sum of TGMP, TGDP and TGTP. The inhibition of the production of these metabolites by allopurinol at high levels was too great resulting in very low values. At 250 μM allopurinol with MP only C was able to produce appropriate concentrations of the sum of TGMP, TGDP and TGTP at 250 μM allopurinol with MP (Figure 5.8). Model C also showed a higher concentration of the sum of TGMP, TGDP and TGTP when a time course was simulated with the combination of 10 μM MP and 100 μM allopurinol than with 10 μM MP and 1 mM allopurinol all reaching similar concentrations to 10 μM MP with no allopurinol (Figure 5.9). This presents another suggestion that this may be a reasonable model as TGNs are active MP metabolites and as 100 μM allopurinol increases the sensitivity of cells to MP whereas 1 mM allopurinol decreases the effects of MP. However, model C predicts lower meTIMP, meTIDP and meTITP in cells treated with higher allopurinol concentrations with MP; given that these metabolites are also cytotoxic, yet cells treated with

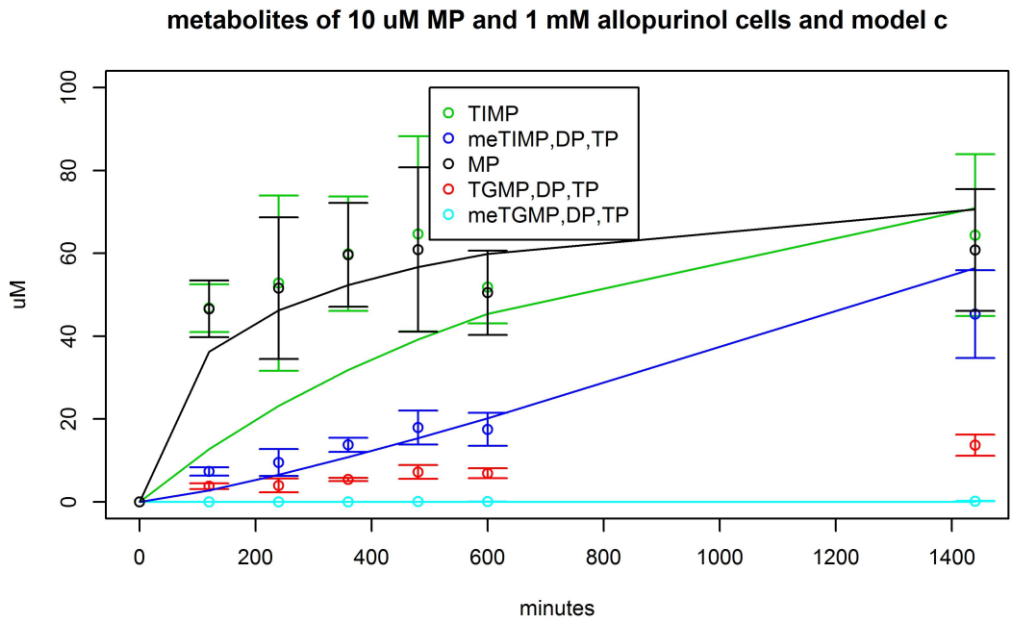
combination of drugs are less sensitive than cells treated with MP and lower concentrations of allopurinol, this may be less likely.


metabolites of 10 uM MP cells and model c
TIMP
meTIMP,DP,TP
MP
TGMP,DP,TP
meTGMP,DP,TP
uM
0
200
400
600
800
1000
0
200
400
600
800
1000
1200
1400
minutes

a


metabolites of 10 uM MP and 250 uM allopurinol cells and model c
TIMP
meTIMP,DP,TP
MP
TGMP,DP,TP
meTGMP,DP,TP
uM
0
20
40
60
80
100
0
200
400
600
800
1000
1200
1400
minutes

b



c

Figure 5.7 (previous 2 pages) Graphs showing the results for each measured metabolite from cultured cells point mean and bars 1 SD (n=3) at **a** 0, **b** 250 and **c** 1000 µM allopurinol with 10 µM M with time course simulations for each drug combination by model C as lines. For each section metabolites are as follows **black** MP, **green** TIMP, **red** TGMP,TGDP,TGTP combined, **blue** meTIMP, meTIDP and meTITP combined and **pale blue** meTGMP, meTGDP and meTGTP.

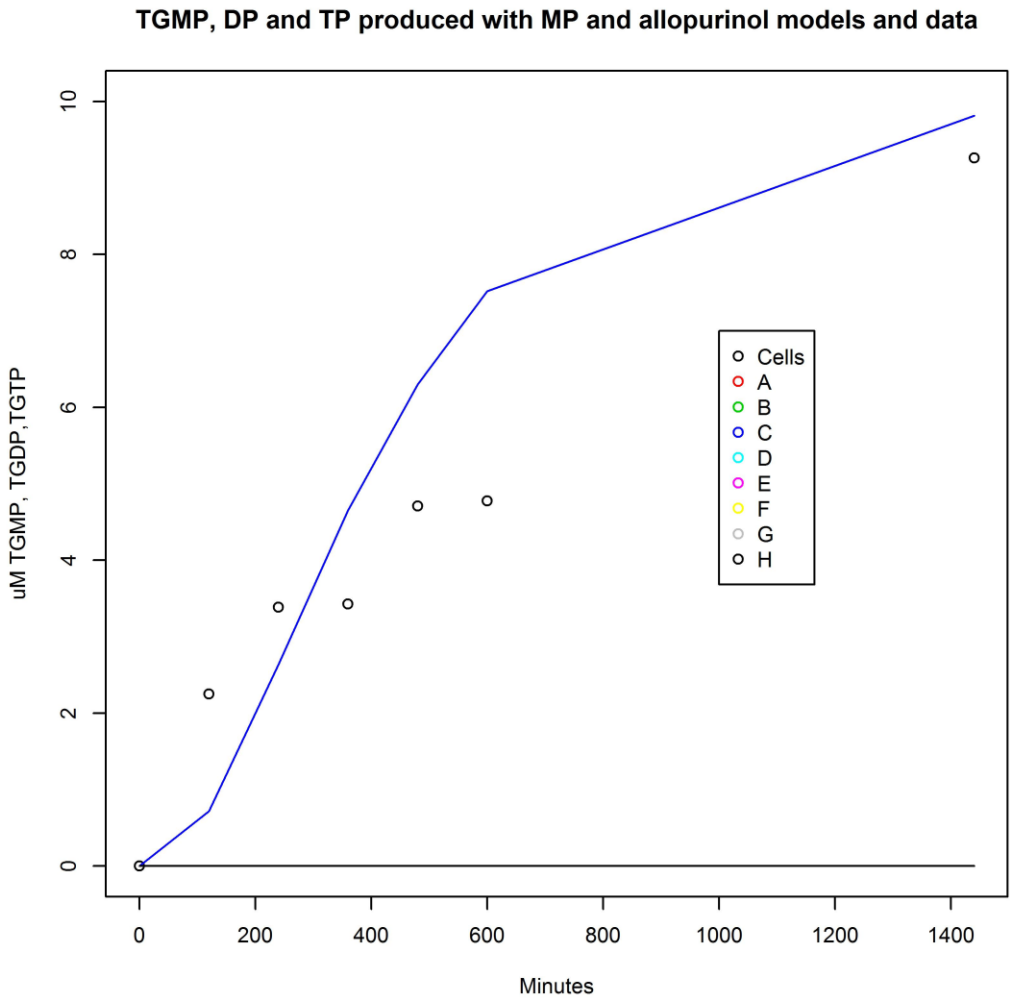


Figure 5.8 TGMP, TGDP and TGTP data from cultured cells points (mean n=3) and simulation of all model variants with 10 µM MP and 250 µM allopurinol, only data from cultured cells and model c (**blue**) visible.

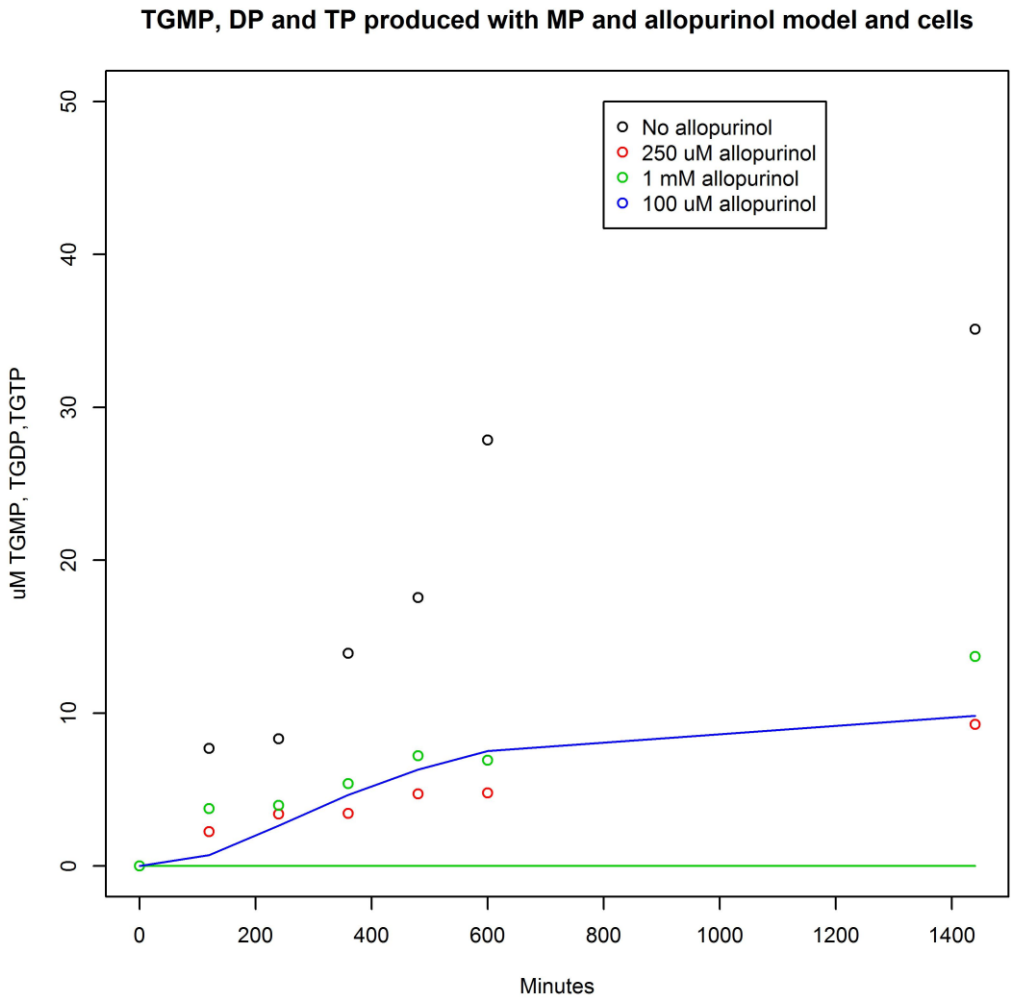


Figure 5.9 TGMP,TGDP and TGTP data from cultured cells points (mean n=3) and simulation of model variant C as lines data created using 10 µM MP with 1 mM allopurinol (**green**), 100 µM allopurinol (**blue** model only), 250 µM allopurinol (**red**) or no allopurinol (**black**) all units µM and minutes.

5.4 Discussion

5.4.1 LC-MS-MS analysis of cell culture data

The LC-MS-MS method developed in Chapter 3 detects thiopurine metabolites in both PBMCs and RBCs from patients treated with thiopurine drugs. The results described in 3.3.4 show that it is also suitable to detect thiopurine metabolites in T-ALL derived cells in culture. This method is more sensitive than previous methods used to detect meTGMP as this was undetectable in cells treated with MP in similar previous cell culture studies (Coulthard *et al.*, 2002) but is detectable at low levels by this method.

Cells grown with 1 mM and 250 µM allopurinol and 10 mM MP do not show the increase in the ratio of TGNs to methylated metabolites seen in patients treated with a combination of thiopurine and allopurinol (Sparrow *et al.*, 2005; Leung *et al.*, 2009; Brackett *et al.*, 2014). There may be several reasons for this. These cells are were only grown for 24 hours whereas patient samples are typically measured after 12 weeks treatment, possibly changes in metabolite levels are dependent on longer term changes in expression of enzymes responsible for thiopurine metabolism. Also cultured cells are only one cell type and are not RBCs, the cell type in which clinical metabolite measurements are made, or hepatocytes, a cell type in which much first-pass thiopurine metabolism takes place; therefore these cells may not be capable of producing this effect on metabolite levels.

5.4.2 Parameter estimation of initial model

Estimating parameters using CoPaSi produced a model which simulated the time course of metabolites corresponding to experimental data with the constraint that [TXMP] should be at lower concentrations than [TIMP] and higher than [TGMP] (Zimm *et al.*, 1985). It also conformed to data showing that [TUA] should be lower concentration than [TIMP] at early time points (Kalra *et al.*, 2007). The differences between the versions of the model with parameters estimated using either the mean of all of the data from cells treated with MP or with the separate replicates of the same data showed that the estimation with the mean produced a better fit, by the measure of sums of squares, to the data used for estimation. This is due to the variability between the separate repeats of the data, meaning that fitting all of the data is not possible with a deterministic model. Deterministic modelling simulates the outcomes of reactions according

to a system of ODEs with the outcome of each reaction being guaranteed for a given set of initial conditions. Stochastic modelling uses an estimate of the probability of a reaction occurring in order to determine whether or not that reaction should progress for each model time-point this results in different outcomes each time the model is simulated, therefore hundreds or thousands of model simulations are run and the outcome of these assessed. This is a more realistic representation of biological reactions that may be unlikely to happen and is able to reflect the variability seen in biological data. Given the high level of biological variability in this data set it may be appropriate to model this system stochastically.

The results of time course simulations (Figure 5.3) from the two model versions were similar in spite of several parameters, such as the Vmax of GMPS, varying by 10 or more fold between the two versions. This suggests insensitivity to these parameters, however a more formal sensitivity analysis would be useful. A sensitivity analysis varies various parameters on the model and then measures the magnitude of the effect on model output; parameters which cause small changes in output are those with low sensitivity. This would be useful information when focussing future studies. Parameters which vary little between model variants, indicating that they are possibly sensitive, such as the Km and Vmax for HGPRT, the rate of incorporation of TGNs into DNA and the uptake of MP into cells may be more important to study in order to improve the model and also as they may be key to the metabolism of thiopurines. Given that variation in CNTs causes MP resistance (Fotoohi *et al.*, 2006a) it seems reasonable that the model parameter representing this reaction is sensitive to changes. It is generally held that IMPDH is the rate limiting reaction in the production of GMP or TGMP from IMP or TIMP (Magasanik *et al.*, 1957); therefore, it is reasonable that the model is likely to be insensitive to the reaction rate of GMPS, a subsequent reaction in this pathway.

5.4.3 The effect of TPMT variation

The model was able to replicate qualitatively, but not quantatively, the effect of increasing TPMT activity on [tDNA] in cells treated with equitoxic doses of MP (Coulthard *et al.*, 2002) (Figure 5.4 a). Given that these results were based on different cell lines equitoxic doses may not be an appropriate test for the accuracy of the model. The model was able to produce qualitatively similar

results with a MP to those described at equitoxic doses of 1.8 and 8 µM MP. [MeTIMP] increases and [tDNA] levels decrease in cells with high TPMT activity compared to normal cells. This suggests that the model is reasonably able to replicate other data on increasing TPMT activity.

The model was also able to qualitatively replicate the effect of lowering TPMT activity in Jurkat cells on meMP (Figure 5.4d) (Misdaq *et al.*, 2012), however the effect is larger in the model. The effect of lowering TPMT activity on TGN concentration in these cells was a non-significant reduction in TGN levels (Misdaq *et al.*, 2012). This is surprising given the effect of low TPMT levels in patients (Lennard *et al.*, 1997; Appell *et al.*, 2013). This effect is not matched in the model, where a large increase in TGN concentration occurs, as would be expected from patient data. This suggests that although the model may not reproduce the situation in Jurkat cells it may be relevant to other systems. TPMT activity is higher in Jurkat cells, which lack MTAP activity, than those with increased MTAP activity (Coulthard *et al.*, 2011), and this produced a decrease in TGNs in MTAP positive Jurkat cells. MOLT-4 have MTAP activity and therefore they would be expected to have lower initial TPMT activity and lower normal TGNs possibly causing the effect of decreasing TPMT activity to be different, as was seen. It may be more appropriate to compare the effect of lowered TPMT in a cell line more similar to MOLT-4s. It may also be useful to include the effect of the cofactor S-adenosyl-methionine and its metabolism by MTAP in a future version of the model.

There is not yet either a knock down or induced TPMT system available in MOLT-4 cells for a true comparison to be made. The model performs reasonably well, compared to currently available cell culture and patient data. Given the known differences between the cells in which data for the model building was collected and those which produced the data used for model testing the effect of cofactors on the reactions of TPMT may be important to the effect of TPMT levels on cellular metabolism of thiopurines. The differences between the model as it stands and the other available data may indicate areas of importance which the model should address.

5.4.4 Assessing the potential mechanisms of allopurinol action on MP metabolism using model variants

None of the model variants was able to fit all of the data. The model variants which fitted the data best, C and A, both included the competitive inhibition of HGPRT by allopurinol (Table 5.3). This is a plausible mechanism given that MP, HGPRT's endogenous substrate HX and allopurinol are all structurally related and may therefore be able to bind the active site of HGPRT. Although patient data show that HGPRT activity is increased after chronic use of a combination of AZA and allopurinol (Seinen *et al.*, 2011), this could be due to increased expression of HGPRT to compensate for inhibition by allopurinol. Such an effect was not included in the model because the model was designed to address changes over shorter time scales. The possibility could be investigated experimentally by measuring the protein levels of HGPRT in patients before and during treatment with this drug combination.

Model C does not show a decrease in the ratio of [meTIMP, meTIDP and meTITP] to [TGMP, TGDP and TGTP] when treated with allopurinol combined with MP. As this was not present in this data this is an accurate reflection of the available data. Further experiments with other drug combinations are needed to determine if this effect occurs in cell culture cells, possibly with a different combination of allopurinol and MP. For this reason model A which includes an inhibition of TPMT by TX and shows this effect is less likely to be true for this data set.

Model C shows an increase in TGNs with 100 μM allopurinol and 10 μM MP compared to 1 mM allopurinol with 10 μM MP; this matches drug sensitivity data from MOLT-4 cells shown in Chapter 2. This was the only model to show this effect. All other models had very low TGNs with any allopurinol and this may support the predictions that HGPRT inhibition and inhibition of MP uptake by allopurinol are important mechanisms for the effects of allopurinol on MP metabolism.

Further data collection is needed to test mechanisms by which allopurinol might interact with MP metabolism. The current models suggest that HGPRT inhibition by allopurinol and TPMT inhibition by TX may both be important, but that inhibition of MP uptake cannot be ruled out as model F (just MP uptake inhibition) was the second best-fitted model. It may be possible to fit both the

original model and the models with allopurinol better if more metabolites were measured. A method for measuring TGN incorporation into DNA by LC-MS/MS is under development and this may enable better fitting of reaction parameters by establishing how many TGNs are lost by this route. It may also be possible to improve the measurement of TX by LC-MS/MS, currently not reported due to high background, and therefore establish the excretion of MP by XO and also to measure the effect of allopurinol on XO activity. Even with current methods it may be possible to distinguish between models by producing data with more different combinations of MP and allopurinol, including 10 μM MP with 100 μM allopurinol, given that only model C produces simulation results that reflect the increase in sensitivity to MP with low-dose allopurinol in MOLT-4 cells. These data could either be compared to the current model output or parameter estimation could be repeated with new data.

5.5 Conclusions

1. A model of MP metabolism has been produced able to model MP metabolism and the effect of TPMT on MP metabolism
2. This model is unable to definitely show which proposed mechanism of action allopurinol has on MP metabolism. It suggests that HGPRT inhibition may be important but does not rule out inhibition of MP uptake

Chapter 6. Conclusions

6.1

Thiopurines are valuable clinical drugs but their cellular and clinical effects are dependent on metabolism within the body. Thiopurine metabolism is not a linear biochemical pathway and the potential outputs in terms of metabolites with distinct mechanisms of toxicity will vary with different expression levels of enzymes and different thiopurine drugs and combinations with other drugs. Therefore, a systems approach is required to understand how thiopurines affect cell behaviour. This was approached by building a model which predicts how the concentrations of thiopurine metabolites within cells relate to drug combination and dose, and how these change over time. The model was developed using MP as the thiopurine drug as this has more complex modes of metabolism compared to TG. A T-ALL derived cell line was used to obtain quantitative experimental measurements of metabolite concentrations on which to base parameter estimates and assess model performance. The model was then used to investigate the effect of combining MP with allopurinol, a clinical drug which affects thiopurine metabolism but in unexpected ways.

6.1.2 Drug sensitivity in cell culture (Chapter 2)

MOLT-4 cells are sensitive to cytotoxicity by the thiopurines AZA and MP, with ED 50's of 6 µM and 3 µM respectively, in agreement with previous literature (Fotoohi *et al.*, 2006a). Febuxostat, a non-purine XO inhibitor is mildly cytotoxic to MOLT-4, Jurkat MTAP $^{-/-}$ and MTAP $^{+/+}$ cells. Allopurinol and oxypurinol, purine analogue XO inhibitors, are not cytotoxic to MOLT-4 and Jurkat cells.

High concentrations, around 1 mM of allopurinol reduce the sensitivity of MOLT-4 cells to MP, increasing the ED 50 from 3.21 µM to 42.23 µM. Lower concentrations of allopurinol, 100 µM or 10 µM, slight but not significantly increase the sensitivity of MOLT-4 cells to MP.

6.1.3 Novel LC-MS/MS method for detecting thiopurine metabolites (Chapter 3)

An LC-MS/MS method capable of detecting 11 thiopurine metabolites and allopurinol has been developed. It is sensitive and reliable enough to detect 9 thiopurine metabolites in both RBCs and PBMC from patients. This method involves less pre-processing than previously developed methods (Lennard, 1987; Dervieux and Boulieu, 1998c; Hofmann *et al.*, 2012; De Nicolo *et al.*,

2014) this will hopefully reduce variability in results as it has been shown that methodological differences are responsible for differences in detection of TGNs by different methods (Shipkova *et al.*, 2003). This reduced pre-processing also allows detection of the mono, di and tri phosphate forms of TGNs and methylated metabolites to be detected individually. This is in contrast to almost all previous methods (Lennard, 1987; Dervieux and Boulieu, 1998c; De Nicolo *et al.*, 2014), most of which rely on acid hydrolysis or enzymatic digestion to remove phosphate groups. These methods produce thioguanosine from a combination of all TGNs and me MPR (De Nicolo *et al.*, 2014) or 4-amino-5-(methylthio)carbonyl imidazole from methylated metabolites (Dervieux and Boulieu, 1998c; Dervieux and Boulieu, 1998b). This pooling of metabolites reduces the information that can be gained about each sample, whether clinical or from cell culture cells. MP and TIMP were also detectable but with slightly less reliability than required for a clinical method. The inclusion of MP, TIMP and allopurinol makes this method able to detect a greater range of thiopurine metabolites and related drugs than previously published methods (Zimm *et al.*, 1985; Hofmann *et al.*, 2012), although the method described by Zimm *et al* (Zimm *et al.*, 1985) was able to detect TXMP which due to a lack of standard this method could not. The ability to measure TXMP would improve estimation of model parameters especially those for IMPDH and GMPS. However, this method was able to provide quantitative data covering many of the metabolites of MP to inform the computational model. Fransson *et al.* (Fransson *et al.*, 2006; Fransson *et al.*, 2007) found that modelling thiopurine metabolism using data for only the two usually reported pooled metabolites, thioguanosine and me MPR, was difficult therefore this new LC-MS/MS method has been important to the success of the computational modelling in this project.

This method was unable to detect the endogenous purines with adequate reliability. Therefore ATP and GTP data were not suitable either for clinical samples or for collection of quantitative data for a computational model; this restricted the scope of the computational model to thiopurine metabolism alone and did not include any interactions with endogenous purine metabolism.

LC-MS/MS data showed that 1 mM and 250 µM allopurinol reduced the concentration of MP metabolites, including TGNs, in cells treated with 10 µM MP. This may explain the reduction in sensitivity to MP given that TGN

incorporation into DNA is important to cell death following thiopurine treatment (Lee and Sartorelli, 1981; Elion *et al.*, 1988; Bohon and de los Santos, 2003).

6.1.4 Approaches for modelling MP metabolism (Chapter 4 and 5)

Various approaches to computational modelling of thiopurine metabolism were attempted. The initial stages for the development of the model of thiopurine metabolism was based on a model of endogenous purine metabolism by Curto *et al* (Curto *et al.*, 1998b). A model combining endogenous purine and thiopurine metabolism could not be justified by either previously available data or data that was collected using the novel LC-MS/MS method. The novel LC-MS/MS method did not produce consistent data about endogenous purine concentrations. This may be due to natural variation in ATP and GTP levels in cells or due to cross-talk from other substances with the same mass and chromatographic characteristics. Therefore a model of only MP metabolism was built in CoPaSi (Hoops *et al.*, 2006).

Metabolite measurements taken over 24 hours after 10 µM MP treatment of MOLT-4 cells were used to estimate the parameters of this model not determined by the literature. This model could qualitatively reproduce the effects of TPMT induction on incorporation of TGNs into DNA and levels of methylated metabolites compared to previously published data (Coulthard *et al.*, 2002). These data were from a different cell line to that modelled and so this was a good result. A comparison of time course simulation data from the model reflecting inhibition of TPMT on TGN and methylated metabolite concentrations showed a qualitative match of methylated metabolite concentrations but no match for TGNs (Misdaq *et al.*, 2012). Given differences in the cell lines used this is not unreasonable, as the cell line in which the data were collected had been shown to lack MTAP whilst the cell line modelled does not and this affects TPMT activity (Coulthard *et al.*, 2011).

Overall, this appears to be a successful model of MP metabolism that would be suitable for further investigations into the effects of varying activities of enzymes of thiopurine metabolites. However, it must be noted that this model has only had parameters estimated with data obtained from one cell line, MOLT-4 cells. This limits conclusions drawn from the model to this cell line, as has been shown by the quantitative differences between the model and previously reported data from other cell lines. One potential use of the model is to compare

the parameters after re-estimating parameters using data sets from other cell lines with different sensitivities to MP. This would allow exploration of how thiopurine metabolism affects sensitivity to thiopurines. A similar approach using a model of the effects of TGN incorporation by Panetta *et al.* (Panetta *et al.*, 2006) explored differences in sensitivity to thiopurines due to cellular response to TGN incorporation into DNA. The model does not reflect whole body metabolism as it relies on data from only one cell line and does not take account of transport of thiopurine drugs and metabolites between compartments. This can be seen in the differences between MOLT-4 cell chromatograms, and chromatograms of RBCs and PBMCs from a patient (Figure 3.2 and Table 3.4). Most strikingly neither cell type from the patient shows much TIMP, whereas in MOLT-4 cells this is a very abundant metabolite. There is also a difference in the relative abundances of the mono, di and tri phosphate forms of the metabolites between both cell types from patients and cultured cells.

The model is also limited in terms of timescale as it uses only data from the first 24 hours after MP treatment, and, as it does not take account of cell division, further testing would be needed to assess the utility of the model beyond this time point.

The model will be useful in complementing the data regarding thiopurine metabolism in cell lines already available and will form the basis on which more complex models can be developed.

6.1.5 Mechanism of interaction between allopurinol and MP metabolism (Chapter 2 and 5)

Metabolite measurements after treatment with 1 mM or 250 µM allopurinol in combination with 10 µM MP showed lower concentrations of all thiopurine metabolites, except for MP, than 10 µM MP alone. This could explain the decreased sensitivity to MP of MOLT-4 cells in the presence of high doses of allopurinol. By reducing the amount of active metabolites cell death is reduced. There was no decrease in the ratio of methylated metabolites to TGNs. This suggests that at these particular concentrations of drug and at these time points, MOLT-4 cell line data does not reflect clinical data where studies show that that allopurinol combined with low doses of AZA or MP reduces the concentration of methylated metabolites compared to TGNs (Sparrow *et al.*,

2005; Leung *et al.*, 2009; Brackett *et al.*, 2014). However, clinical data from patients treated with low dose AZA/allopurinol is generally reported after 12 weeks of treatment and the thiopurine metabolites are measured in the surrogate compartment, the RBCs. Therefore the fact that this effect was not observed in cell culture cells over 24 hours, in an enclosed system, is perhaps not surprising.

These metabolite data were used to estimate kinetic parameters of 8 versions of the model of MP metabolism and its proposed interactions with allopurinol. None of the versions of the model produced fitted much better than any other, based on sums of squares assessments of the fit of the models, all of which were similar. As discussed below there may be other methods of assessing the fit of the data to the various model structures. However the two models which performed slightly better than any others both included inhibition of HGPRT activity by allopurinol. One of these model variants, C, also produced a better fit for TGMP, TGDP and TGTP concentration with 250 μM allopurinol with MP. These metabolites are important to the effects of MP therefore the ability to fit this particular aspect of the data set may indicate that this model is better. A more sophisticated method of weighting the importance of data would be likely to improve the discrimination between the models than is possible using the default method available in CoPaSi, a mean squares method. For example, the application of more formal model selection metrics such as Akaike information criterion (Akaike, 1974) which assess across whole time course profiles and have the advantage of penalising models with large numbers of parameters.

This indication that allopurinol might inhibit HGPRT may contradict clinical data showing increased HGPRT activity with chronic allopurinol and AZA use (Seinen *et al.*, 2011). However, the clinically-reported effect could be due to transcriptional induction over a longer timescale of HGPRT to compensate for lack of activity; such an induction of enzyme expression may not be detected in shorter experiments. This effect could be tested experimentally by assessing the levels of HGPRT in patient cells before and after treatment with allopurinol and AZA or in cultured cells over a longer time. Currently the model variants that included allopurinol inhibiting MP uptake into cells cannot be formally excluded. The data do not support alterations to the ratio of methylated

metabolites to TGNs in short term treatment with high allopurinol compared to MP, therefore the model is unable to confirm the inhibition of TPMT by TX seen in cell lysate studies (Blakera *et al.*, 2012), however further data need to be collected before a concentration dependency of this effect can be ruled out.

This part of the project has only obtained preliminary results, requiring further experimental data for a more accurate model to be produced. However these preliminary results suggest interesting lines of future inquiry.

6.2 Future Work

6.2.1 Future work on cell culture

The results showing the effect of relatively high concentrations of allopurinol on MP action have only been shown in MOLT-4 cells, and therefore need to be confirmed with both lower concentrations of allopurinol and in other cells, e.g. Jurkat cells. Currently, to determine if this is a cell line specific, the effect of combining AZA and allopurinol is being studied in A549 (lung cancer cells) by Dr Sally Coulthard and preliminary results from Mr Joshua O'Hara (Masters Student; personal communications) suggest that the efficacy of AZA is reduced by high concentrations of allopurinol in A549 cells and that the concentration of active metabolites is lower in cells exposed to this treatment.

In addition to these studies the effect of allopurinol on both AZA and TG metabolism should be investigated to confirm if the same phenomenon is present, a reduced efficacy with high concentrations of allopurinol and an increased efficacy with low concentrations of allopurinol. Investigating the other XO inhibitors, febuxostat and oxypurinol, a metabolite of allopurinol, to confirm if they also affect thiopurine metabolism would also be useful. If it is found that febuxostat has a similar effect to allopurinol when combined with thiopurine this may be of particular clinical relevance as some patients suffer from hypersensitivity to allopurinol but not febuxostat (Ramasamy *et al.*, 2013). A recent clinical paper suggests that febuxostat may also modify thiopurine metabolism to increase the proportion of TGNs to methylated inosine metabolites (Dore *et al.*, 2014).

Together all these data would further inform the model to understand the mechanism of action of combining XO inhibitors with thiopurines.

Allopurinol and oxypurinol are only mildly cytotoxic, and are designed as such, therefore ED50 data are not obtainable but determining how much drug would be required to inhibit XO activity to 50% would be useful for fixing the inhibition constants in the model. This would require the optimisation or development of a XO activity assay.

6.2.2 Future Work on LC-MS/MS

Ideally more thiopurine metabolites should be measured, for example me MP and TX. These were tuned for with the MS, but the fragments initially studied produced very high background levels. This can be addressed by selecting and optimising the detection of alternative fragments, thus allowing the study of XO inhibition as these data would likely improve the models ability to fit a parameter to XO activity and also inhibition by allopurinol of XO and potential inhibition of TPMT by TX.

In addition, measuring TUA, the final product of XO metabolism, would also help inform future models. However TUA is very insoluble, and could not be determined by HPLC, and no LCMS/MS determination was attempted.

Measuring TXMP would enable the contribution of IMPDH and GMPS to thiopurine metabolism to be distinguished. Measurement of TXMP was made by HPLC however only a small amount was obtained as a gift (Dr Sofie Haglund) and it is not commercially available, therefore this could not be included in the LC-MS/MS method.

Further refinement of the measurement of allopurinol and development of a method to measure its metabolite oxypurinol would also be helpful in order to investigate further their interaction with thiopurines.

To increase the accuracy of the computational model it will be important to measure TGNs that have been incorporated into DNA with greater sensitivity and less pre-processing than with currently available methods, which generally include the addition of a flurophore to the TGN in DNA (Coulthard *et al.*; Hogarth *et al.*, 2008) or use a carbon-13 isoform as a standard for deoxythioguanosine (Wang and Wang, 2010b). This would enable more specific fixing of the rate of incorporation of TGNs into DNA which would allow other model parameters to be better fixed. Also this would allow this model to be used to investigate the effects of incorporated TGNs in DNA on cytotoxicity.

Furthermore improving the method for detecting endogenous purines would allow the downstream effects of methylated metabolites to be included in this model allowing more investigations into the methods of thiopurine cytotoxicity.

The ratios of the mono, di and tri phosphates of thiopurines detected in cells were variable and therefore the phosphatase and kinase reactions causing this were not modelled. Mr Philip Berry and Dr Sally Coulthard are investigating the effects of sample processing on this problem.

Also RBC and PBMC samples from patients and cell culture samples all show different metabolite profiles (Figure 3.2). Intercellular transport may be the cause of some of these differences (Fang *et al.*, 2010) and it would be interesting to model this. To explore these effects a post-doctoral project has begun to compare the metabolite profiles in RBCs and PBMCs incubated together or separately with MP and to use these data to expand the existing model of MP metabolism to a model of whole blood metabolism of MP.

6.2.3 Future work towards a model combing thiopurine and endogenous purine metabolism

There are several areas of interest in the interactions of thiopurines and endogenous purines. For instance, a more detailed exploration of the effects of meTIMP on DNPS (Mario *et al.*, 1993). This would require more reliable data about the concentrations of endogenous purines before and after treatment with thiopurine drug. Comparison of cell growth in HX containing and HX free media and the effect of thiopurines on endogenous purine metabolism would help shed light on the importance of inhibition of DNPS by thiopurine metabolites.

MTAP an enzyme involved in adenosine salvage also affects the efficacy of thiopurine drugs (Coulthard *et al.*, 2011). In order to improve our understanding of these effects the interaction between DNPS and thiopurines would first need to be modelled. A model of the spermine pathway including MTAP would be useful to gain a deeper understanding of the role of DNPS, this could possibly draw on an existing model of polyamine metabolism (Rodríguez-Caso *et al.*, 2006). Interestingly MTAP also appears to have an effect on TPMT (Coulthard *et al.*, 2011) activity and the nature of this effect could be modelled to suggest lines of further inquiry.

To model the effects of thiopurines on endogenous purine metabolism and *vice versa* detailed data about the interactions between endogenous purines and thiopurines with common enzymes is needed. A method using nuclear magnetic resonance spectroscopy data to determine kinetic parameters for carbon metabolism reactions has recently been described (Eicher *et al.*, 2012) and would be very useful to help determine the requisite enzyme activities.

6.2.4 Future work on the interaction of allopurinol with thiopurine metabolism

The conclusions that could be drawn about the interaction of allopurinol with MP were limited; more data need to be collected and a more sophisticated method of simultaneously fitting multiple data sets to multiple model structures needs to be used to discriminate better between the proposed model structures.

Metabolite data at other concentrations of MP and different ratios of MP to allopurinol could help to better fix the parameters of the proposed models. Particularly if the clinical effect of allopurinol on thiopurine metabolism, reduced methylated metabolites, is concentration dependent, other ratios and concentrations of MP and allopurinol should be investigated.

TX concentrations in particular, would be useful to improve the fit of the models as this would inform the parameters of XO activity and inhibition and also the potential effect if TX on TPMT. This could improve the distinction between different model variants.

It may be more appropriate to fit the multiple versions of the model to multiple data sets using different software. For example the MatLab extension PottersWheel (Maiwald and Timmer, 2008), which has the facility to perform these comparisons directly which CoPaSi (Hoops *et al.*, 2006) does not have. PottersWheel would also allow an identifiability analysis to be performed (Hengl *et al.*, 2007). Model identifiability describes the extent to which parameters can be uniquely assigned given a model structure and the available data, but because the model was built in a programme not designed to perform identifablity analysis this question has not yet been addressed. However a method has recently been published allowing this to be performed in CoPaSi although it uses hidden features of CoPaSi (Schaber, 2012). Identifiability analysis would allow improved targeting of future data collection experiments.

Despite PottersWheel being a more appropriate programme for these tasks it was not used during this project as CoPaSi was adequate for the initial phases of model building and fitting and although both programmes allow the import and export of SBML models they both modify them for use. Due to time constraints modifying and revalidating the model in PottersWheel was not feasible.

6.3 Overall conclusion

A model able to replicate data about the metabolism of MP in cultured cells was produced. This model was able replicate the effect of TPMT on MP metabolism previously described in the literature.

A novel method of detecting thiopurine metabolites in cell culture samples and patient blood has been developed.

It has been shown that high concentrations of allopurinol in cultured cells reduce the efficacy of MP by reducing the concentrations of active metabolites. Low concentrations of allopurinol non-significantly increase the sensitivity of MOLT-4 cells to MP, further experiments are needed to determine what effect this drug combination has on metabolite concentrations.

This project has not been able to distinguish the mechanism by which allopurinol interacts with MP metabolism by using a computational model. However preliminary data from the model suggests that this effect may depend on inhibition of HGPRT by allopurinol, an effect not previously seen.

The model of MP metabolism has given some insights into the clinically relevant combination of thiopurine and allopurinol. The model will provide a basis for further studies about this and other aspects of MP metabolism. This will benefit future patients by better targeting the dosing of this drug.

Appendix A

SBML Model definition of the first model described in Chapter 5

```
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11:28 with libSBML version 3.4.1. -->
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version="4">
  <model metaid="COPASI1" id="Model_1" name="New Model">
    <annotation>
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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syntax-ns#" xmlns:dc="http://purl.org/dc/elements/1.1/"
xmlns:dcterms="http://purl.org/dc/terms/"
xmlns:vCard="http://www.w3.org/2001/vcard-rdf/3.0#"
xmlns:bqbiol="http://biomodels.net/biology-qualifiers/"
xmlns:bqmodel="http://biomodels.net/model-qualifiers/">
        <rdf:Description rdf:about="#COPASI1">
          <dc:creator>
            <rdf:Bag/>
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          <dcterms:created rdf:parseType="Resource">
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      <functionDefinition id="function_1" name="Henri-Michaelis-
Menten (irreversible)">
        <math xmlns="http://www.w3.org/1998/Math/MathML">
          <lambda>
            <bvar>
              <ci> substrate </ci>
            </bvar>
            <bvar>
              <ci> Km </ci>
            </bvar>
            <bvar>
              <ci> V </ci>
            </bvar>
            <apply>
              <divide/>
              <apply>
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```
                <times/>
                <ci> V </ci>
                <ci> substrate </ci>
              </apply>
              <apply>
                <plus/>
                <ci> Km </ci>
                <ci> substrate </ci>
              </apply>
            </apply>
          </lambda>
        </math>
      </functionDefinition>
    </listOfFunctionDefinitions>
    <listOfUnitDefinitions>
      <unitDefinition id="time">
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        </listOfUnits>
      </unitDefinition>
      <unitDefinition id="substance">
        <listOfUnits>
          <unit kind="mole" scale="-6"/>
        </listOfUnits>
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name="compartment" size="2e-012">
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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            </rdf:RDF>
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name="extracellular" size="0.07">
        <annotation>
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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08T14:08:30Z</dcterms:W3CDTF>
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              </dcterms:created>
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    </compartment>
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compartment="compartment_1" initialConcentration="0">
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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    <species metaid="COPASI5" id="species_3"
name="tGMP_tGDP_tGTP" compartment="compartment_1"
initialConcentration="0">
      <annotation>
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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compartment="compartment_1" initialConcentration="0">
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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              </dcterms:created>
            </rdf:Description>
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compartment="compartment_1" initialConcentration="0">
      <annotation>
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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            </rdf:Description>
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compartment="compartment_1" initialConcentration="0">
      <annotation>
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
            <rdf:Description rdf:about="#COPASI8">
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            </rdf:Description>
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    </species>
    <species metaid="COPASI9" id="species_7" name="tX"
compartment="compartment_1" initialConcentration="0">
      <annotation>
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          <rdf:RDF xmlns:dcterms="http://purl.org/dc/terms/"
xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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                </rdf:Description>
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            </rdf:Description>
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      <species metaid="COPASI10" id="species_8" name="TUA"
compartment="compartment_1" initialConcentration="0">
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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            </rdf:RDF>
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        </annotation>
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      <species metaid="COPASI11" id="species_9" name="metIMP"
compartment="compartment_1" initialConcentration="0">
        <annotation>
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            <rdf:RDF xmlns:dcterms="http://purl.org/dc/terms/"
xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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      <species metaid="COPASI12" id="species_10"
name="metGMP_metGDP_metGTP" compartment="compartment_1"
initialConcentration="0">
        <annotation>
          <COPASI xmlns="http://www.copasi.org/static/sbml">
            <rdf:RDF xmlns:dcterms="http://purl.org/dc/terms/"
xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
              <rdf:Description rdf:about="#COPASI12">
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                    <dcterms:W3CDTF>2014-02-
10T10:06:12Z</dcterms:W3CDTF>
                  </rdf:Description>
                </dcterms:created>
              </rdf:Description>
            </rdf:RDF>
          </COPASI>
        </annotation>
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      </species>
      <species metaid="COPASI13" id="species_11" name="meMP"
compartment="compartment_1" initialConcentration="0">
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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                </dcterms:created>
              </rdf:Description>
            </rdf:RDF>
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compartment="compartment_1" initialConcentration="0">
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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            </rdf:RDF>
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      <species metaid="COPASI15" id="species_13" name="TGout"
compartment="compartment_1" initialConcentration="0">
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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                </dcterms:created>
              </rdf:Description>
            </rdf:RDF>
          </COPASI>
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      <species metaid="COPASI16" id="species_2" name="MPout"
compartment="compartment_2" initialConcentration="10">
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        <annotation>
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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            </rdf:RDF>
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        </annotation>
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    </listOfSpecies>
    <listOfReactions>
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uptake" reversible="false">
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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            </rdf:RDF>
          </COPASI>
        </annotation>
        <listOfReactants>
          <speciesReference species="species_2"/>
        </listOfReactants>
        <listOfProducts>
          <speciesReference species="species_1"/>
        </listOfProducts>
        <kineticLaw>
          <math xmlns="http://www.w3.org/1998/Math/MathML">
            <apply>
              <ci> function_1 </ci>
              <ci> species_2 </ci>
              <ci> Km </ci>
              <ci> V </ci>
            </apply>
          </math>
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            <parameter id="Km" name="Km" value="0.121008"/>
            <parameter id="V" name="V" value="1.79258e-011"/>
          </listOfParameters>
        </kineticLaw>
      </reaction>
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      <reaction metaid="COPASI18" id="reaction_2" name="HGPRTi"
reversible="false">
        <annotation>
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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                  </rdf:Description>
                </dcterms:created>
              </rdf:Description>
            </rdf:RDF>
          </COPASI>
        </annotation>
        <listOfReactants>
          <speciesReference species="species_1"/>
        </listOfReactants>
        <listOfProducts>
          <speciesReference species="species_5"/>
        </listOfProducts>
        <kineticLaw>
          <math xmlns="http://www.w3.org/1998/Math/MathML">
            <apply>
              <times/>
              <ci> compartment_1 </ci>
              <apply>
                <ci> function_1 </ci>
                <ci> species_1 </ci>
                <ci> Km </ci>
                <ci> V </ci>
              </apply>
            </apply>
          </math>
          <listOfParameters>
            <parameter id="Km" name="Km" value="548.223"/>
            <parameter id="V" name="V" value="43.2477"/>
          </listOfParameters>
        </kineticLaw>
      </reaction>
      <reaction metaid="COPASI19" id="reaction_3" name="IMPDH"
reversible="false">
        <annotation>
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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        </COPASI>
      </annotation>
      <listOfReactants>
        <speciesReference species="species_5"/>
      </listOfReactants>
      <listOfProducts>
        <speciesReference species="species_4"/>
      </listOfProducts>
      <kineticLaw>
        <math xmlns="http://www.w3.org/1998/Math/MathML">
          <apply>
            <times/>
            <ci> compartment_1 </ci>
            <apply>
              <ci> function_1 </ci>
              <ci> species_5 </ci>
              <ci> Km </ci>
              <ci> V </ci>
            </apply>
          </apply>
        </math>
        <listOfParameters>
          <parameter id="Km" name="Km" value="4815.39"/>
          <parameter id="V" name="V" value="20.0001"/>
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      </kineticLaw>
    </reaction>
    <reaction metaid="COPASI20" id="reaction_4" name="GMPS"
reversible="false">
      <annotation>
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      <listOfReactants>
        <speciesReference species="species_4"/>
      </listOfReactants>
      <listOfProducts>
        <speciesReference species="species_3"/>
      </listOfProducts>
      <kineticLaw>
        <math xmlns="http://www.w3.org/1998/Math/MathML">
          <apply>
            <times/>
            <ci> compartment_1 </ci>
            <apply>
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                <ci> species_4 </ci>
                <ci> Km </ci>
                <ci> V </ci>
              </apply>
            </apply>
          </math>
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            <parameter id="Km" name="Km"
value="76855800000000"/>
            <parameter id="V" name="V" value="353686000000"/>
          </listOfParameters>
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      </reaction>
      <reaction metaid="COPASI21" id="reaction_5" name="DNA
incorporation" reversible="false">
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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          </COPASI>
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        <listOfReactants>
          <speciesReference species="species_3"/>
        </listOfReactants>
        <listOfProducts>
          <speciesReference species="species_6"/>
        </listOfProducts>
        <kineticLaw>
          <math xmlns="http://www.w3.org/1998/Math/MathML">
            <apply>
              <times/>
              <ci> compartment_1 </ci>
              <ci> k1 </ci>
              <ci> species_3 </ci>
            </apply>
          </math>
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            <parameter id="k1" name="k1" value="0.0393604"/>
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        </kineticLaw>
      </reaction>
      <reaction metaid="COPASI22" id="reaction_6" name="XOMP"
reversible="false">
        <annotation>
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            </rdf:RDF>
          </COPASI>
        </annotation>
        <listOfReactants>
          <speciesReference species="species_1"/>
        </listOfReactants>
        <listOfProducts>
          <speciesReference species="species_7"/>
        </listOfProducts>
        <kineticLaw>
          <math xmlns="http://www.w3.org/1998/Math/MathML">
            <apply>
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              <ci> compartment_1 </ci>
              <apply>
                <ci> function_1 </ci>
                <ci> species_1 </ci>
                <ci> Km </ci>
                <ci> V </ci>
              </apply>
            </apply>
          </math>
          <listOfParameters>
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            <parameter id="V" name="V" value="1.14089"/>
          </listOfParameters>
        </kineticLaw>
      </reaction>
      <reaction metaid="COPASI23" id="reaction_7" name="XOtX"
reversible="false">
        <annotation>
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xmlns:rdf="http://www.w3.org/1999/02/22-rdf-syntax-ns#">
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Publications and Abstracts

Poster: A Computational Model of the Action of Xanthine Oxidase on Thiopurine Drugs: Azathioprine,6-Mercaptopurine and 6-Thioguanine Sarah McGarrity, Chris Redfern, Daryl Shanley, Sally Coulthard “Systems Biology of Human Disease” Heidelberg, Germany, May 2012

Poster: “Building a dynamic model of thiopurine metabolism” Sarah McGarrity, Philip Berry, Chris Redfern, Daryl Shanley, Sally Coulthard “International Study Group in Systems Biology” Durham, United Kingdom, September 2014

Paper (submitted): “A novel liquid chromatography-mass spectrometry method for measuring thiopurine metabolites in white blood cells” Phil Berry, Sarah McGarrity, Azhar Ansari, Christopher P. F. Redfern & Sally A. Coulthard submitted to Analytical Chemistry, August 2014

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