Modelling the effects of inhibitors of guanine nucleotide synthesis: implications for studies of cellular differentiation pathways

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Abstract

Mizoribine induces the differentiation of promyelocytes by an unknown mechanism that relies on compromised guanine nucleotide synthesis. I have found that mizoribine also perturbs adenosine nucleotide levels in HL-60 promyelocytes, particularly ATP. To reconcile these observations with the known actions of mizoribine I have adapted an existing model of human purine metabolism composed as an S-system familiar from Biochemical Systems Theory. Mizoribine’s actions were then simulated and compared with experimental data.

Introduction

MZB (mizoribine, CAS 50924-49-7) is an immunosuppressant sometimes valuable in the prevention of kidney rejection after transplant [1]. However, MZB also has an unexplained property: it induces the differentiation of promyelocytes, the type of cells characteristic of promyelocytic leukaemia [2]. Promyelocytes are essentially leucocytes that have failed to make a final commitment to either granulocyte or monocyte fate. MZB induces differentiation towards granulocytes and its effect correlates with changes in cellular guanine nucleotide levels, principally GDP and GTP, and can be reversed by treatment with guanine or guanosine [3]. Once transported inside cells, MZB is converted into MSP (MZB 5-phosphate), a potent competitive inhibitor of IMPDH (inosine monophosphate dehydrogenase) and, to a lesser extent, GMPS (GMP synthetase), two enzymes which catalyse sequential steps in GMP synthesis from IMP. Since there is a salvage pathway leading to GMP synthesis that uses guanine or guanosine and bypasses IMPDH and GMPS, then MZB’s differentiating effects are thought to be explained by compromised guanine nucleotide synthesis. Interestingly, other promyelocyte differentiating agents also perturb guanine nucleotide synthesis e.g. DMSO [4], and it is thought that this common change may be sensed by a G-protein-dependent signalling pathway. However this G-protein sensor has not been identified and the only major insight has been the exclusion of Ras as a candidate, despite its suspected role in the transformed phenotype of promyelocyte lines [4]. Differentiation to granulocytes requires MZB to act in the first 24 h and cannot be reversed later; obviously some unknown event at the early stages commits the cells to express characteristic adhesion machinery and other complex components important for granulocyte physiology [2].

Several model systems are in common use for the exploration of promyelocyte differentiation. One of the most useful cell types are HL-60 cells, since they can be directed to either granulocytes or monocytes by a range of agents. For example, ATRA (all-trans retinoic acid) drives HL-60 cells towards granulocytes by engaging nuclear receptors, and its mechanism is relatively well understood compared with that of DMSO, which has similar effects but for less well described reasons. Interestingly, these two different agents encourage different routes (trajectories in a very high dimensional space that describes the dynamics of gene expression) to the final differentiated state, but the final outcome is essentially the same (e.g. expression profiles differ at 24 h but are very similar at 168 h; [5]). MZB is not the only IMPDH inhibitor that induces the differentiation of HL-60 cells. Mycophenolic acid, a non-competitive inhibitor of IMPDH and another agent useful in preventing transplant rejection, converts HL-60 cells towards monocytes (but with substantial apoptosis; [6]).

During the investigation of HL-60 cells as a model of leukemic promyelocyte differentiation, I found that MZB also perturbs levels of adenosine nucleotides, particularly ATP. This indicates that, although the enzymatic locus of MZB’s action may be assigned to guanine nucleotide metabolism, it is nonetheless capable of inducing changes in the wider purine metabolic system. Changes in adenine nucleotides report the energy status of the cell through AMP kinase. In turn, signalling mechanisms that rely on mTOR (mammalian target of rapamycin) integrate this energy status with amino acid availability and regulate protein synthesis [7], a process required for differentiation. Hence these concurrent effects on adenine nucleotides cannot be ignored. Notably,
adenosine and adenine do not have the same effects as guanosine or guanine in reversing the differentiating action of MZB despite their capacity to feed adenine nucleotide pathways. Therefore adenine nucleotide metabolism may be a secondary modulator for the primary guanine nucleotide trigger. An initial investigation of the literature describing the biochemical basis of MZB’s actions produced a majority of reports featuring very linear and uncomplicated metabolic pathways. Further inspection revealed that this simplicity is inappropriate, since guanine nucleotide metabolism is part of a rich web of inter-conversion and feedback control [8]. Clearly the problem of discovering how MZB produces its effects on cellular phenotypes would benefit from a more complete understanding of its impact on both guanine and adenine nucleotide metabolism. To approach this problem, I have adapted and expanded an existing mathematical model of human purine metabolism [9] composed as an S-system familiar from Biochemical Systems Theory. This model was used to simulate decreases in IMPDH and GMPS activity and rescue through the salvage pathway. The model was then compared with experimental data obtained using HL-60 promyelocytes.

Results and discussion

Survey of existing models of human purine metabolism

There are several existing models of human purine metabolism and so I investigated whether any of these might form a useful foundation. Some of the most complete and comprehensively documented models are those created by Curto et al. [9–11]. Their three distinct mathematical models feature systems of coupled differential equations representing rate laws expressed in three different ways: (i) ‘complemented Michaelis–Menten’ kinetics; (ii) GMA (general mass action) kinetics and (iii) an S-system formulation derived from the GMA forms. This diversity of formalisms seems surprising but is actually extremely prudent as it allows the consideration of differences that spring solely from the mathematics and not the underlying biochemistry and the associated modelling assumptions. Traditionally, biochemical rate laws are expressed using classical MMHK (Michaelis–Menten–Henri kinetics), which provide rational functions containing well-recognized constants such as $V_{\text{max}}$ and $K_m$ and similar parameters that are often extremely hard to determine in vivo. Powerful alternative approaches take advantage of Taylor’s theorem and the ability to model any function, including biochemical rate laws, with a power law, and this approach has been popularized in Biochemical Systems Theory [12]. Power laws enjoy useful advantages over standard MMHK approaches e.g. traditional constants need not be explicitly determined, but they also have their own drawbacks and so Biochemical Systems Theory and MMHK methods should be seen as complementary.

Biochemical Systems Theory uses two principle types of models: GMA, which can serve as a bridge to MHMK; and S-systems, which are unique. GMA and S-systems differ only in that fluxes are represented as discrete input–output terms for each reaction needed in the rate law but are aggregated into single input and output terms in S-systems. Since Curto et al’s [9–11] original models of human purine metabolism were given most effectively as both GMA and S-systems, it was desirable to build my new models in these same forms. For the purposes of the present paper, only the properties of the S-system model are analysed and compared with data.

Adaptation of existing models to incorporate MZB’s action

In the models of purine metabolism made by Curto et al. [9–11], many of the metabolites that change upon treatment with MZB or guanosine, or both, are aggregated into pools e.g. adenosine/AMP/ADP/ATP and GMP/GDP/GTP [9], and this is inappropriate for the required model. Figure 1 shows two parts (A and B) detailing my novel expansion of these pools to describe the necessary metabolites and their inter-relations explicitly. The appropriate additional metabolic links are drawn from the KEGG PATHWAY database (as of January 2010, [8]) and the regulatory arcs are those extensively documented by Curto et al. [9–11] and included in their models. The destinations of some metabolic pathways and regulatory arcs are omitted from

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**Figure 1** Metabolic pathways added to the previous models of purine metabolism

The models created by Curto et al. [9–11] contain many metabolites aggregated as pools and this is unsuitable for a model to examine changes induced by MZB. (A) Expansion of the guanine nucleotide pool (GMP/GDP/GTP) and (B) the adenine nucleotide pool (adenosine/AMP/ADP/ATP) as described in the text. Solid arrows (→) indicate enzyme-catalysed metabolic conversions. Dashed arrows (→→→) indicate activating regulation. Dashed and dotted arrows (→→→) indicate inhibitory regulation. Ado, adenosine; Gua, guanine; Guo, guanosine; Hx, hypoxanthine; Ino, Inosine; PRPP, phosphoribosylpyrophosphate; SAM, S-adenosyl methionine; S-AMP, adenylosuccinate; Xa, xanthine; prefix d indicates the deoxy form.
The Figures for clarity, only the origins are shown. As an illustration of the modelling process, Figure 2 shows the new relations required to model GMP metabolism overtly and also indicates the form of these relations expressed as a rate law taken from my intermediate GMA model and the derived S-system form. The operation of expanding the earlier model increases the number of dependent variables to be modelled, and therefore the number of differential equations in the system from 16 to 23. In the final S-system model there are an additional 22 rate constants (paired production and removal constants) and 24 reaction kinetic orders (again in pairs for balance) and this model was chosen for further investigation and evaluation. Estimated values for these parameters were obtained from literature sources or some initial experiments made in permeabilized HL-60 cells by studying the rates of conversion of $^3$H radio-labelled adenine or guanine nucleotides (G.M.H. Thomas, unpublished work). The model arrived at steady-state metabolite values, many of which were in reasonable agreement with the earlier models. In the next step, parameters to describe the competitive inhibition of IMPDH (types 1 and 2) with respect to IMP, and GMPS with respect to XMP ($K_i \leq 10$ nM and $10 \mu$M respectively [1,13]), by M5P were incorporated into the rate laws describing the change of XMP and GMP. Using a conservative assumption that the intracellular concentration of M5P is 10% of the concentration of MZB in the incubation medium, the model system was perturbed and new steady-state concentrations of metabolites were estimated. It was sometimes difficult to find new steady-states using expected initial values of metabolite concentrations and so in these cases near steady-state estimates were taken at extended time points. In later work using principled and systematic investigation of the model parameter space it was found that the model became both more stable and representative with initial concentrations of metabolites different from those used by Curto et al. [9–11]. To discover whether these values were reasonable, some of them were re-estimated in intact or permeabilized HL-60 cells (G.M.H. Thomas, unpublished work).

**Notable modelling assumptions and limitations**

The model contains many assumptions, but for simplicity I will highlight only a few. First, the diffusion of metabolites within the cell is modelled as instantaneous and there is no significant metabolic compartmentalization or other local build-ups of metabolites. Therefore no delays need to be incorporated into the equations. Secondly, all-important components are within the system boundary and so all-important inter-relations (metabolic or regulatory) are described in the model. Thirdly, no modelled precursors (ribose 5-phosphate and P$_i$) are depleted or exhausted in the course of modelled experiments, they remain fixed independent variables. As an adjunct, all important components not explicitly described are similarly universally available (water, ions, cofactors etc.). Fourthly, changes in cell volume during the cell cycle and their potential influence on the concentrations of model components are ignored. Lastly, the levels of expression or the specific activity of the modelled enzymes does not change over time.

Besides these assumptions, there are important intrinsic limits to the model. First, S-systems have known complications where metabolic branch points are modelled or aggregated [14] and purine metabolism features many such branch points. Secondly, most metabolic models are accurate for infinitesimally small experimental perturbations and this includes S-systems. Hence approximations of large perturbations may be relatively unreliable and the extent of this unreliability is not known. Lastly, S-systems do not cope
The final model was implemented using PLAS software with both Taylor and BDF/LSDA type solvers (http://www.dgb.fc.ul.pt/docentes/aferreira/plas.html). (A) The sensitivity analysis for the kinetic orders in the model; only absolute values are given for clarity. The notation is the usual form used in S-systems: $g_{i,j}$ reveals the metabolite being influenced ($i$) and the influencing agent ($j$). Hence the kinetic orders in the sensitivity analysis are: $g_{42,41}$, adenosine on AMP; $g_{43,42}$, AMP on adenosine; $g_{43,44}$, AMP on ADP; $g_{42,43}$, ADP on AMP; $g_{44,43}$, ADP on ATP; $g_{43,44}$, ATP on ADP; $g_{62,81}$, GDP on GTP; $g_{61,82}$, GDP on GMP; $g_{63,83}$, GDP on GTP; $g_{92,83}$, GTP on GDP; $g_{61,83}$, GTP on GMP. (B) Measured compared with predicted nucleotides, the performance of the S-system model of purine metabolism in response to challenge by MZB. The measured metabolite levels (Real) were compared with predicted levels (Model) expressed as either nmoles/10^6 HL-60 cells or molar ratios.

**Sensitivity analysis for terms added to create the new model**

A preliminary analysis of the response of the system to changes in the values of the newly added system parameters was undertaken. As an example, Figure 3(A) shows the sensitivity of both guanine and adenine nucleotides to the estimated values of kinetic orders, each value may appear several times in the S-system model either in the production terms ($g_{i,j}$) or the degradation terms ($h_{i,j}$, results not shown), and this constraint underpins some aspects of ensuring flux balance. It should be noted that terms of the form $g_{i,j}$ are synonymous with the reaction rate elasticities well established in other approaches to modelling the kinetics of multi-enzyme systems. For simplicity, the absolute values of the sensitivities are shown in Figure 3(A). Notably, several of the magnitudes were greater than 1, indicating that further attention must be paid to these components and their values.

**Comparing the model with reality**

To examine the agreement between model and experiment, HL-60 cells were treated with 50 μM MZB for various times and the levels of GDP, GTP, ADP and ATP were determined by HPLC. To confirm the impact of the drug on the promyelocytes, the characteristic changes in morphology and CD11b expression (and reversal by guanosine) were routinely monitored by electron microscopy and flow cytometry respectively and confirmed to be present. Notably, biphasic changes were seen in the elevation of ATP concentration and the decrease in GTP. It is not known whether the return towards the nucleotide levels found in untreated control cells represents the metabolic or other inactivation of MZB or an induced homeostatic response of the HL-60 cellular system. Given the extended time-courses of differentiation and nucleotide changes, I compared the real and predicted nucleotide concentrations at steady-state (or a suitably extended time point). The Table in Figure 3(B) shows a typical set of results comparing GDP, GTP, ADP and ATP levels. Since the GDP/GTP ratio is thought to influence the activity of G-proteins and the ADP/ATP ratio indicates the energy status of the cell, then these two ratios are also described. The most striking feature of this comparison is that the model data is rarely in strict numerical agreement with values of nucleotides measured in HL-60 cells. However in all cases, the direction of change in nucleotide levels induced by MZB, guanosine or a combination of both agents was correctly predicted. Hence my expanded model of Curto et al. [9–11] has qualitative predictive power but needs improvements for accurate quantitative projections.

**Future work**

Since my model requires improvement, it is reasonable to speculate on how this might be achieved. First, the ‘parent’ model [9] is a ‘whole body’ model for human purine metabolism and not just leucocytes or their progenitors. It is not certain whether all of the enzymes described in the original model and incorporated into my expansion are expressed in HL-60 cells, and so this needs to be confirmed. Secondly, the model contains the implicit assumption that the levels or activity of the modelled enzymes do not change significantly over the course of a differentiation experiment. However, although this is adequate for an initial study, it has an obvious weakness – when HL-60 cells (and all cells) differentiate, then by definition their gene expression is altered significantly. Probably, induction or suppression of enzymes might lead to changes in the overall structure of the model or of parameters or both. Thirdly, the fluxes through enzymes with sensitive kinetic orders (Figure 3A) need to be re-examined at different differentiation conditions.
time points along with absolute concentrations of their metabolites. Lastly, the new models should be re-evaluated for performance with these new flux and concentration parameters.

**Conclusion**
The established models of Curto et al. [9–11] can be used as a foundation for expanded models of purine metabolism with the explicit metabolites described. These expanded models can provide good qualitative predictions of the impact of guanine nucleotide synthesis inhibitors on the purine metabolic system found in HL-60 cells.

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