Pharmacokinetics and pharmacodynamics of thiopurines in an in vitro model of human hepatocytes: insights from an innovative mass spectrometry assay.

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Running title: Thiopurines biotransformation in hepatocytes

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Abstract

AIM:

To apply an innovative LC-MS/MS method to quantify thiopurine metabolites in human hepatocytes and to associate them to cytotoxicity.

METHODS:

Immortalized human hepatocytes (IHH cells) were treated for 48 and 96 hours, with 1.4×10^{-4} M azathioprine and 1.1×10^{-3} M mercaptopurine, concentrations corresponding to the IC_{50} values calculated after 96 hours exposure in previous cytotoxicity analysis. After treatments, cells were collected for LC-MS/MS analysis to quantify 11 thiopurine metabolites with different level of phosphorylation and viable cells were counted by Trypan blue exclusion assay to determine thiopurines *in vitro* effect on cell growth and survival. Statistical significance was determined by analysis of variance.

RESULTS:

Azathioprine and mercaptopurine had a significant time-dependent cytotoxic effect (p-value ANOVA = 0.012), with a viable cell count compared to controls of 55.5% and 67.5% respectively after 48 hours and 23.7% and 36.1% after 96 hours; no significant difference could be observed between the two drugs. Quantification of thiopurine metabolites evidenced that the most abundant metabolite was TIMP, representing 57.1% and 40.3% of total metabolites after 48 and 96 hours. Total thiopurine metabolites absolute concentrations decreased over time: total mean content decreased from 469.9 pmol/million cells to 83.6 pmol/million cells (p-value ANOVA = 0.0030). However, considering the relative amount of thiopurine metabolites, TGMP content significantly increased from 11.4% cells to 26.4% (p-value ANOVA =0.017). A significant association between thiopurine effects on viable cell counts could be detected only for MeTIMP: lower MeTIMP concentrations were associated with lower cell survival (p-value ANOVA = 0.011). Moreover, the ratio between MeTIMP and TGMP metabolites directly correlated with cell survival (p-value ANOVA = 0.037).

CONCLUSION:

Detailed quantification of thiopurine metabolites in a human hepatocytes model provided useful insights on the association between thioguanine and methyl-thioinosine nucleotides with cell viability.

Core tip.

Accurate quantification of 11 thiopurine metabolites in human hepatocytes after azathioprine and mercaptopurine exposure was achieved by applying an innovative LC-MS/MS method. Among them, relevant concentrations were found for 5 metabolites: TIMP, TGMP, MeTIMP, TGTP, and TGDP. Overall, it was observed that, considering their absolute levels, concentrations of thiopurine nucleotides decreased over time. By contrast, considering their relative amount, thioguanine nucleotides are the only group of metabolites that increases over time. Intriguingly, the ratio between the concentration of MeTIMP and TGMP metabolites was significantly associated with cell survival, as potentially representative of the balancing between intracellular catabolic and anabolic processes.

INTRODUCTION

The thiopurines azathioprine and mercaptopurine are antimetabolite drugs widely used for their immunosuppressive action. These drugs are employed to treat several chronic autoimmune pathologies, to avoid rejection after organ transplantation and also as anti-leukemic agents [1]. Thiopurines are prodrugs that require complex conversion (Figure 1) to be activated to thioguanine nucleotides (TGNs).

Figure 1. Metabolism of azathioprine (AZA) and mercaptopurine (6-MP). 6-Me-TGDP, 6-methylthioguanosine 5'-diphosphate; 6-Me-TGMP, 6-methylthioguanosine 5'-monophosphate; 6-Me-TGTP, 6-methylthioguanosine 5'-triphosphate; 6-Me-TIDP, 6-methylthioinosine 5'-diphosphate; 6-Me-TIMP, 6-methylthioinosine 5'-monophosphate; 6-Me-TITP, 6-methylthioinosine 5'-triphosphate; 6-MMP, 6-methylmercaptopurine; 6-TGDP, 6-thioguanosine 5'-diphosphate; 6-TGMP, 6-thioguanosine 5'-monophosphate; 6-TGTP, 6-thioguanosine 5'-triphosphate; 6-TIDP, 6-thioinosine 5'-diphosphate; 6-TIMP, 6-thioinosine 5'-monophosphate; 6-TITP, 6-thioinosine 5'-triphosphate; 6-TU acid, 6-thiouric acid; 6-TXMP, 6-thioxanthine 5'-monophosphate; GMPS, guanosine monophosphate synthetase; GST, glutathione-S-transferase; HPRT, hypoxanthine guanine phosphoribosyltransferase; IMPDH, inosine monophosphate dehydrogenase; ITPA, inosine triphosphate pyrophosphatase; K, kinase; TPMT, thiopurine S-methyltransferase; XO, xanthine oxidase.

This conversion is catalyzed by several enzymes of purine salvage pathway [2]. The cytotoxicity of thiopurines on leukemic blasts and lymphocytes occurs principally through the incorporation of active TGNs in nucleic acids [3] but also by the inhibition of *de novo* purines synthesis mainly due to methyl thioinosinic metabolites [4] and of specific signaling pathways in activated lymphocytes [5]. However, despite the proven efficacy of these drugs, some patients present severe adverse effects such as pancreatitis, hepatitis, and leukopenia [6]. Interindividual variability in thiopurine effects is principally due to their complex cellular metabolism, that involves genes that display genetically determined polymorphic activity, such as thiopurine-methyltransferase (TPMT), resulting in the accumulation of toxic levels of metabolites in patients with reduced TPMT activity [7]. Therefore a quantitative analysis is fundamental to monitor patients' metabolites concentration [7-9]. Several methods have been developed, using high performance liquid chromatography combined with detection by UV (HPLC-UV) or mass spectrometry (HPLC-MS). However, most of these methods are able to quantify only some of the thionucleotides produced intracellularly during thiopurine administration [10-12]. In particular, the majority of methods are unable to distinguish the level of phosphorylation of the thionucleotides. Triphosphate nucleotides have a more important role in the lympholytic activity than the respective monophosphates, therefore it would be important to ascertain the amount of mono-, di- and tri-phosphate nucleotides [11,13,14]. Several studies have shown that the level of phosphorylation of thioguanine metabolites is an important determinant of thiopurines effects, both clinically and *in vitro* [14-16]. For example, a missense variant (R139C) of nucleoside diphosphate hydrolase (NUDT15), an enzyme that hydrolyzes the thiopurine active metabolites 6-thio-deoxyGTP (6-thio-dGTP) and 6-thio-GTP to monophosphate thionucleotides, can affect thiopurine effect. In particular, NUDT15 ablation potentiated the DNA damage checkpoint and cancer cell death by thioguanine nucleotides, resulting in an increased sensitivity to thiopurines, due to the accumulation of triphosphate TGN [17,18]. Moreover, another important enzyme of thiopurine metabolism, inosine triphosphate pyrophosphatase (ITPA) can influence thiopurines effect by altering the concentration of triphosphate and monophosphate nucleotides. This enzyme catalyzes the pyrophosphohydrolysis of inosine triphosphate to inosine monophosphate. Polymorphisms in ITPA, leading to a deficiency in its activity, affect the concentrations of

6

thiopurine metabolites, resulting in intolerance due to abnormal accumulation of inosine triphosphate [19].

Recently, a highly specific and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for simultaneous quantitation of eleven mono-, di-, and triphosphates of thionucleotides has been developed, allowing to finely quantify individual metabolites in patient erythrocytes [20], instead of the total amount of the phosphorylated forms, as measured by standard methods [11,13,21]. The aim of this study is to apply this innovative mass spectroscopy assay to identify and quantify thiopurine metabolites produced after *in vitro* treatment of human hepatocytes with azathioprine and mercaptopurine and to associate the relevant metabolites concentration with the cytotoxic activity of the drugs.

MATERIALS AND METHOD

Cell cultures

The IHH cell line [22] was maintained in Dulbecco's modified Eagle's medium (DMEM EuroClone, Milan, Italy) high glucose with the addition of 10% fetal bovine serum (Sigma-Aldrich, Milan, Italy), 1.25% L-glutamine 200 mM (EuroClone, Milan, Italy), 1% penicillin 10000 UI/mL (EuroClone, Milan, Italy), streptomycin 10 mg/mL (EuroClone, Milan, Italy), 1% Hepes buffer 1 M (Sigma-Aldrich, Milan, Italy), 0.01% human insulin 10^{-4} M (Sigma-Aldrich, Milan, Italy), and 0.04% dexamethasone 1 mg/mL (Sigma-Aldrich, Milan, Italy). Cell cultures were maintained according to standard procedures in a humidified incubator at 37 °C and with 5% CO_2, and cell passage was performed once a week.

Treatment with thiopurine drugs

IHH cells (1×10^6) were seeded in 25 cm^2 flasks and treated, for 48 and 96 hours, with azathioprine (1.4×10^{-4} M, Sigma-Aldrich, Milan, Italy) and mercaptopurine (1.1×10^{-3} M, Sigma-Aldrich, Milan, Italy) both diluted in NaOH 0.1 M. Control cultures were treated with the same volume of NaOH 0.1 M used for drug treatment (final concentration of NaOH 1.6×10^{-4} M). The concentrations of azathioprine and mercaptopurine correspond to the IC$_{50}$ [23] obtained after a 96 hours thiopurine exposure previously evaluated in IHH cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL, Sigma-Aldrich, Milan, Italy) test.

Processing of samples for mass spectroscopy assay

At the end of incubation period, cells were detached by exposure to a solution of trypsin 0.05% and EDTA 0.02% in PBS (Sigma-Aldrich, Milan, Italy), counted by the Trypan blue dye exclusion assay and collected after centrifugation for 5 minutes at 400xg; the cell pellets were then stored at -80 °C until analysis. Samples were then sent to the collaborating laboratory in Stuttgart (Germany), for quantification of thiopurine metabolites by LC-MS/MS, as previously reported [20]. A mixture of 250 µL of EDTA (50 mM), 15 µL of 30 mg/mL DTT solution, and 10 µL of internal standard working solution (20 pmol/µL [2H3]MeTGMP, 60 pmol/µL [2H3]MeTGDP/ [2H3]MeTGTP, 100 pmol/µL [2H3]MeTIMP, 160 pmol/µL [2H3]MeTIDP/ [2H3]MeTITP, 40 pmol/µL [2H4]TGMP, 80 pmol/µL [2H4]TGTP/[2H4]TGDP) was added to the cell pellet and vortex mixed. Proteins were denatured by heating at 95 °C for 5 min in a water bath, and the samples were subsequently extracted by addition of 50 µL of methanol followed by the addition of 250 µL of dichloromethane with thorough mixing after each step. After centrifugation at 16100 g for 20 min, 5 µL of the supernatant was used for LC-MS/MS analysis as described previously [20]. Metabolites quantification was normalized based on the number of viable cells for each sample and was reported as pmol/millions of viable cells.

Evaluation of cell viability

Once detached, before processing the cells for the mass spectrometry analysis, cell viability was determined on the basis of viable cell counts obtained by the Trypan blue exclusion assay. Results are reported as viable cell counts respect to untreated controls.

Statistical analysis

All data analyses were performed within the R software environment (version 3.2.4) for statistical computing and graphics. Data were analyzed by fitting analysis of variance (ANOVA) models (aov function of the stats package), considering, for the *in vitro* metabolites measurements, each metabolite concentration as the dependent variable and exposure time and drug used as independent variables (corresponding to a two-way ANOVA on a model: metabolite concentration ~ exposure time * drug used); for the *in vitro* cytotoxicity analysis, the percentage of viable cells in comparison to untreated controls was

considered as the dependent variable and metabolites concentrations, exposure time and drug used as the independent variables, both in univariate ANOVA and in a multivariate combined analysis. For each drug, experiments at 96 hours were performed in triplicate, while at 48 hours in duplicate.

RESULTS

IHH cell counts and treatment with azathioprine and mercaptopurine

In order to assess the disposition of thiopurine antimetabolites, an *in vitro* study was carried out on IHH cells (1×10^6) that were seeded in 25 cm² flasks and treated with azathioprine (1.4×10^{-4}M) and mercaptopurine (1.1×10^{-3}M). Cells were collected after 48 or 96 hours of incubation, counted and processed for thiopurine metabolites dosage by the LC-MS/MS assay. Both azathioprine and mercaptopurine induced a significant time-dependent reduction of cell viability (p-value two-way ANOVA for treatment duration effect = 0.046), with 55.5% and 67.5% of viable cell counts as compared to controls after 48 hours, and 28.3% and 33.1% after 96 hours, respectively. No significant differences could be observed between the two drugs (Figure 2).
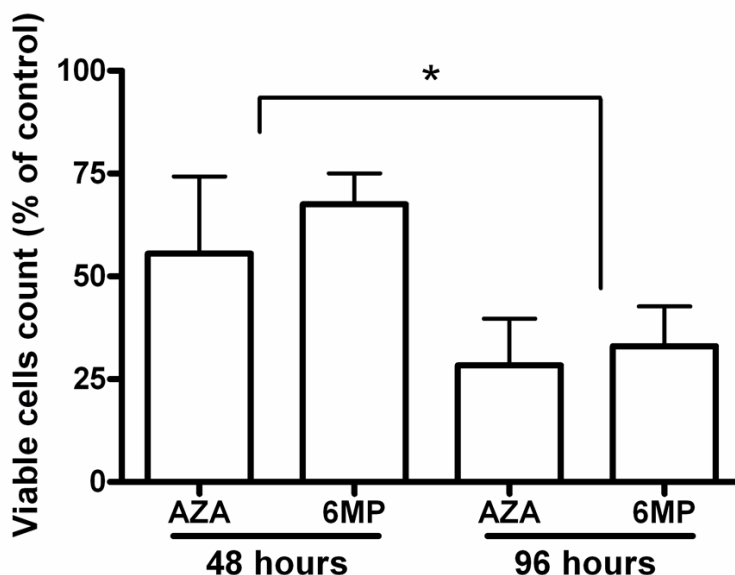

Figure 2. Viable cells count evaluated by Trypan blue dye exclusion assay after 48 and 96 h exposure to 1.4×10^{-4} M azathioprine and 1.1×10^{-3} M mercaptopurine. Results are reported as viable cells count respect to untreated controls. *: p<0.05 (Two-way ANOVA).

Thiopurine metabolites concentrations in IHH cells treated with azathioprine and mercaptopurine

For the thiopurine metabolites, a summary of the measurements is provided in Table 1 for azathioprine and mercaptopurine.

Table 1: average concentration of thiopurine metabolites in IHH cells treated with mercaptopurine and azathioprine for 48 and 96 hours								
Metabolites	48 hours		48 hours		96 hours		96 hours	
	Mercaptopurine		Azathioprine		Mercaptopurine		Azathioprine	
	Concentration (pmol / million cells)	% thiopurine metabolites	Concentration (pmol / million cells)	% thiopurine metabolites	Concentration (pmol / million cells)	% thiopurine metabolites	Concentration (pmol / million cells)	% thiopurine metabolites
TIMP	269.66±170.84	61.7%	339.13±239.43	52.5%	66.80±17.39	48.5%	13.24±5.62	33.1%
TITP	0	0%	0	0%	0	0%	0	0%
TGMP	28.55±1.64	8.7%	72.53±14.27	14.2%	24.82±7.73	17.4%	14.01±4.98	30.8%
TGDP	2.57±0.59	0.83%	10.45±0.43	2.2%	1.58±1.58	1.1%	1.77±1.11	2.6%
TGTP	16.62±12.61	6.3%	53.08±34.98	14.0%	7.82±5.37	5.4%	5.17±4.02	6.5%
MeTIMP	66.88±11.23	21.1%	72.99±6.03	15.9%	35.74±7.48	25.3%	11.46±5.76	20.3%
MeTIDP	0	0%	0	0%	1.38±1.38	0.93%	0	0%
MeTITP	3.18±3.18	1.3%	2.88±2.88	0.84%	1.64±1.64	1.1%	0	0%
MeTGMP	0	0%	0.14±0.14	0.039%	0	0%	0	0%
MeTGDP	0	0%	0.16±0.16	0.047%	0.17±0.17	0.11%	0.20±0.20	0.52%
MeTGTP	0.29±0.29	0.12%	0.78±0.78	0.23%	0.27±0.27	0.18%	0.11±0.11	0.3%
Total thiopurine metabolites	387.73±141.31	100%	552.12±209.17	100%	140.20±46.97	100%	45.95±19.14	100%

Overall, for thiopurine metabolites, a significant difference between azathioprine and mercaptopurine could be observed only for absolute values of TGDP (p-value two-way ANOVA for thiopurine used effect = 0.021), with azathioprine having a higher concentration of this metabolite, considering treatment duration.

The most abundant metabolite was TIMP, representing 47.8% of all thiopurine metabolites, 57.1% after 48 hours and 40.3% after 96 hours. The other classes of thiopurine nucleotides, ranked according to relative abundance in cells, are thioguanine nucleotides (29.8% of total metabolites) and methylthioinosine nucleotides (22.0% of total metabolites); methylthioguanine nucleotides are the less abundant (0.4% of total metabolites).

Considering the total amount of all thiopurine nucleotides, there is an overall decrease over time: total mean content decreases from 469.9 pmol/million cells to 83.6 pmol/million cells (p-value two-way ANOVA for treatment duration effect = 0.0030) after 48 and 96 hours, respectively. A significant decrease of TGMP, TGDP, TGTP, TIMP and meTIMP, the most abundant metabolites (mean intracellular concentration > 5 pmol/million cells) was evident: the most significant decrease was observed for meTIMP, with a reduction of the mean concentration from 69.9 to 21.1 pmol/million cells from 48 to 96 hours (p-value two-way ANOVA for treatment duration effect = 0.0013, Figure 3).
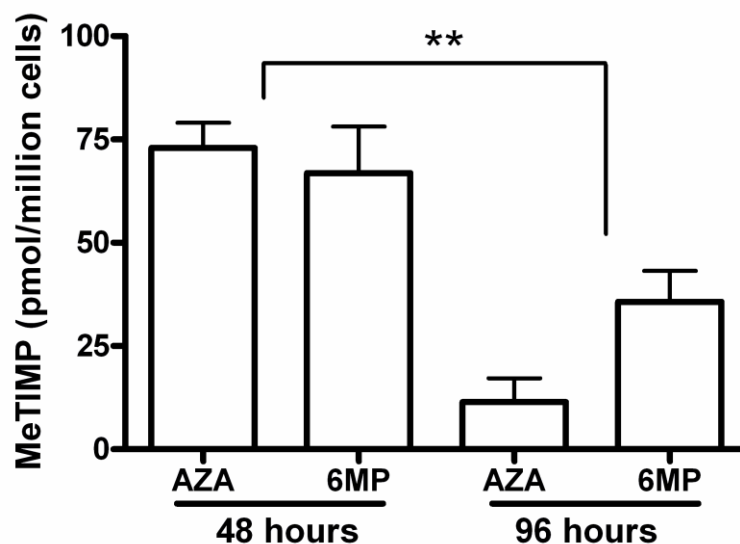

Figure 3. MeTIMP concentrations in IHH cells after 48 and 96 h exposure to 1.4 x 10^{-4} M azathioprine and 1.1 x 10^{-3} M mercaptopurine. Results are reported as pmol/10^6 cells. **: p<0.01 (Two-way ANOVA).

Considering the relative amount of thiopurine metabolites, interestingly, thioguanine nucleotides are the only group of metabolites that proportionally increases: in particular, the percentage of TGMP content significantly increases from 11.4% to 26.4% (p-value two-way ANOVA for treatment duration effect = 0.017, Figure 4).
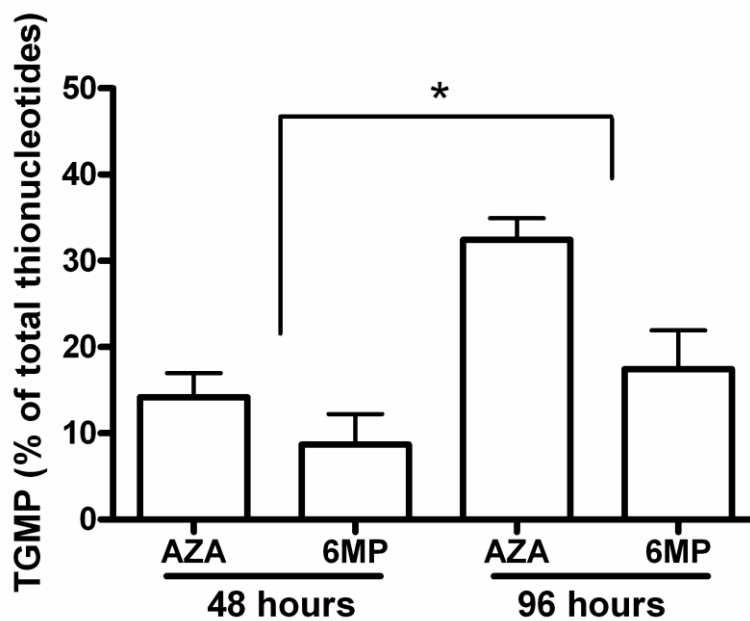

*Figure 4. Relative amount of TGMP in IHH cells after 48 and 96 h exposure to 1.4 x 10^{-4} M azathioprine and 1.1 x 10^{-3} M mercaptopurine. Results are reported as TGMP % with respect to the total amount of thiopurines metabolites. *: p<0.05 (Two-way ANOVA).*

No other significant difference for the relative amount of thiopurine metabolites is evident over time (Table 1).

Association between thiopurine metabolites and cell viability

Analysis of the correlation between the effect of the drugs on cell viability (percentage of live cell counts of treated cells in comparison to untreated controls, determined by Trypan blue dye assay) and the concentration of thiopurine metabolites has considered the most abundant metabolites TIMP, TGMP, MeTIMP, TGTP, and TGDP (average values considering all samples > 5 pmol/million cells). A significant association could be detected in the univariate analysis between MeTIMP concentration and thiopurines cytotoxic

activity, with lower MeTIMP concentrations associated with lower viable cell counts (p-value ANOVA = 0.011, Figure 5).
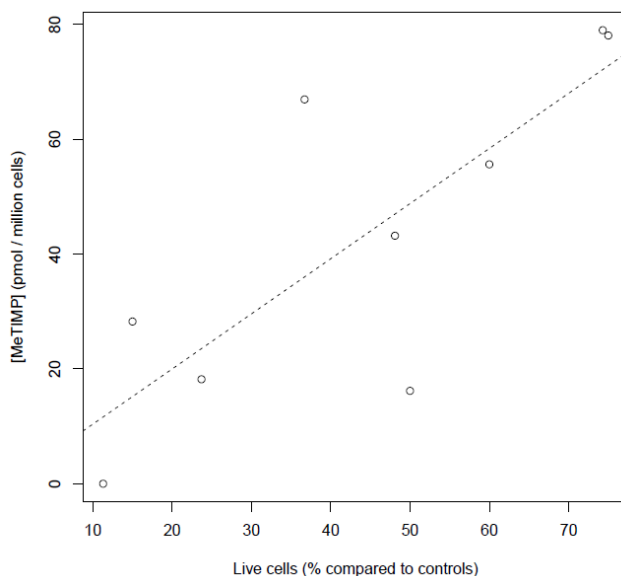

Figure 5. Correlation analysis between MeTIP concentrations in IHH cells and thiopurines cytotoxicity (% of live cell counts of treated cells in comparison to untreated controls, determined by Trypan blue dye assay). Figures contains combined data for azathioprine and mercaptopurine experiments.

No other metabolite was significantly associated with a reduction of the number of viable cells, both considering the total or relative amount of metabolites. An interesting observation is that the ratio between MeTIMP and TGMP metabolites directly correlates with cell viability (p-value ANOVA = 0.037, Figure 6).
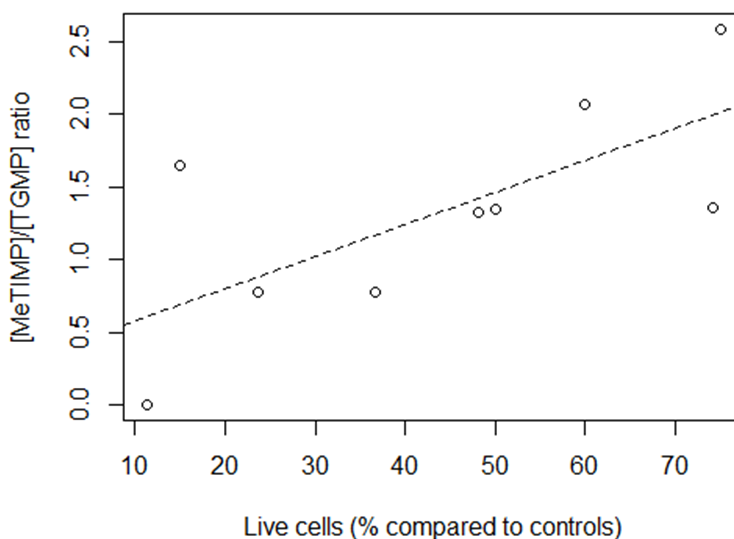
Figure 6. Correlation analysis between the ratio of MeTIP and TGMP concentrations in IHH cells and thiopurines cytotoxicity (% of live cell counts of treated cells in comparison to untreated controls, determined by Trypan blue dye assay). Figures contains combined data for azathioprine and mercaptopurine experiments

A multivariate analysis considering the association between cell growth and MeTIMP concentration, adjusted for the drug used, confirms a significant effect of MeTIMP (p-value ANOVA = 0.0021) and no significant difference between the two drugs (p-value ANOVA = 0.36). Also an analysis considering the two drugs independently confirms an association between cell growth and MeTIMP concentration after mercaptopurine treatment (p-value ANOVA = 1.24×10^{-5}) and a similar trend is present after azathioprine treatment (p-value ANOVA = 0.057). Further multivariate analysis for cell growth, combining treatment duration, drug used and metabolites concentrations did not show any significant effect for MeTIMP or other metabolites.

DISCUSSION

In this work, we apply for the first time an innovative mass spectrometry method to quantify comprehensively thiopurine metabolites produced by a non-tumor model of immortalized human hepatocytes (IHH), after *in vitro* treatment with a concentration corresponding to the IC$_{50}$ of azathioprine and mercaptopurine calculated after 96 hours of exposure. This cell line could be representative of a tissue important for thiopurine biotransformation, especially after oral administration, and potential target of significant adverse effects. In addition, being a non-tumor cell line, results could not be potentially affected by altered metabolic pathways, as in the case of tumor cell lines [22]. Among the 11 metabolites evaluated, 5 (TIMP, TGMP, MeTIMP, TGTP and TGDP) were present in significant amounts, while for the others (TITP, MeTIDP, MeTITP, MeTGMP, MeTGDP, MeTGTP,), very low amounts could be detected. The total absolute amount of thiopurine metabolites decreased between 48 and 96 hours of treatment, an effect which is not dependent on the reduction of viable cells after 96 hours of exposure with respect to 48 hours of exposure, since metabolites quantification was normalized on the basis of the number of viable cells. However, considering the relative amount, for TGMP a significant increase over time could be

detected: this could be related to the fact that thioguanine nucleotides are the final products of anabolic reactions of thiopurine metabolism and may require more time to accumulate in cells [1,24]. Considering the association between thiopurine metabolite concentrations and cell viability during treatment with azathioprine and mercaptopurine, only for MeTIMP a significant association could be observed: MeTIMP concentration was directly associated with cell survival, therefore, cells with higher concentration of this metabolite displayed reduced cytotoxic activity from thiopurines. This result contrasts with previous reports describing MeTIMP as significantly contributing to *in vitro* cytotoxic effects of azathioprine and mercaptopurine, by inhibiting *de novo* purine synthesis [13,25]. Indeed, in our model, considering measurement of thiopurine concentration at two time-points (i.e., 48 and 96 hours) and given the direct correlation between MeTIMP concentration and cell viability, MeTIMP concentration could be a surrogate of treatment length, therefore the association observed could be related to the fact that longer exposure was associated with a reduction in thiopurines cytotoxicity. Multivariate analysis combining treatment duration and thiopurine metabolite concentrations did not show any significant effect, likely because of the limited number of conditions analyzed (only one drug concentration, two time-points) and more detailed studies should be performed. Interestingly, the ratio between TGMP and MeTIMP was also associated with cell survival in our model of human hepatocytes, with a higher ratio associated with reduced cell survival: this could reflect increased activation over time of thiopurines to active thioguanine nucleotides over less cytotoxic methyl-thioinosine nucleotides and in particular MeTIMP.

In the literature, another study[25] evaluated in detail the concentration of some thiopurine metabolites after *in vitro* treatment with mercaptopurine, in human leukemia (CEM) and murine fibroblast (NIH-3T3) cell lines using an HPLC-UV method, which anyway did not discriminate between nucleotides with different levels of phosphorylation. In this paper, metabolites were measured after 24 and 48 hours of exposure to mercaptopurine and a significant decrease over time of total thionucleotides was observed, as in our study. Interestingly, total concentration of thioguanine nucleotides (TGMP+TGDP+TGTP) measured in our hepatic cells (after 48 hours of treatment with 1100 µM mercaptopurine, 91.9 pmol/million cells), considering the dose administered, may be relatively lower than those reported for the leukemia cell line (after 48 hours of treatment with 10 µM

mercaptopurine, 59.6 pmol/million cells) and the fibroblasts cell line (after 48 hours of treatment with 10 µM mercaptopurine, 24.8 pmol/million cells); moreover, concentrations of methylated thionucleotides are much lower in our model system than reported for leukemia and fibroblasts cells. These observations are in agreement with the lower sensitivity of IHH cells to thiopurines (IC_{50} measurable only after 96 hours and equal to 1100 µM) [23] we previously described, compared to CEM and 3T3 cells (IC_{50} at 48 hours respectively 1.5 and 10 µM) [25]. Differently from what was observed in CEM and 3T3, in our work, MeTIMP concentration was directly associated with cell survival, therefore cells with higher concentration of this metabolite displayed reduced cytotoxic activity. This could be due to the fact that, in our model, MeTIMP concentration correlated with exposure time, as stated above. Further studies are already in progress considering the effect of alterations in the concentration of enzymes relevant for thiopurine biotransformation, such as TPMT, on thioguanine nucleotides measured by the innovative mass-spectrometry based assay used in this study.

Previous studies done on human hepatocytes cytosols have shown that, in human liver, mercaptopurine metabolic profile is dependent upon drug concentration. In addition, with concentrations in the millimolar range, several thiopurine metabolites could be detected after 48 hours of exposure, with TIMP as the most abundant, even if no thioguanine nucleotides could be measured [26]. In our study, we confirm TIMP as the most abundant metabolite after *in vitro* treatment even if we could detect, both at 48 and 96 hours, relevant concentrations of thioguanine nucleotides, likely because of the increased sensitivity of our LC-MS/MS approach.

Clinical application of the innovative mass spectrometry method used in our study, measuring thiopurine metabolites in patients' erythrocytes after treatment with thiopurines, showed a different metabolite pattern than our *in vitro* hepatocytes model. In particular, in patients' erythrocytes, methyl thioinosine nucleotides were the most abundant species, while they are very low in our *in vitro* system; on the other hand, TIMP is the highest thionucleotide detectable in our model, while it is among the lowest in patients erythrocytes. These differences could be related to the fact that in hepatocytes thiopurine metabolites are directly produced from the orally administered drug, while erythrocytes accumulate active

metabolites produced by other cells, mainly hepatocytes. The differences observed between patients' erythrocytes and our hepatocyte *in vitro* model may be related to the fact that metabolism of thiopurines in cellular systems might also be dependent on the substrate concentration. This has been described for leukemia cells by Liliemark et al., which reported that the conversion of TIMP to TGN decreases at mercaptopurine concentrations above 5 μM *in vitro* [4].

Recent studies have shown that also the microbiota present in the intestinal flora could have a role in the conversion of the thiopurine drugs into therapeutically active thiopurine nucleotides (TGN) [27]. This can improve significantly the effect of thiopurines in the treatment of patients with IBD and more studies could be dedicated to develop *in vitro* models considering also the contribution of microbiota, besides relevant tissues for thiopurines effects and toxicity, such as intestine, liver, white blood cells and pancreas.

CONCLUSION

In this work, we assessed for the first time the *in vitro* pharmacokinetics of azathioprine and mercaptopurine in a non-tumor immortalized human hepatic cell line, by an innovative mass spectrometry assay that allows a comprehensive quantification of thiopurine metabolites, comprising also the different levels of phosphorylation. After treatment with azathioprine and mercaptopurine, it is possible to quantify relevant concentrations of 5 metabolites (TIMP, TGMP, MeTIMP, TGTP, and TGDP), with TIMP as the most abundant, among 11 which are produced during the treatment with these drugs. The concentrations of thiopurine nucleotides decreased over time. The ratio between the concentration of MeTIMP and TGMP metabolites was significantly associated with cell survival, as potentially representative of the balancing between intracellular catabolic and anabolic processes. More studies should be performed to model *in vitro* chronic exposure to thiopurines, considering other relevant tissues such as human pancreas, evaluating the effects of alterations in candidate genes related to thiopurines pharmacokinetics, to further understand biotransformation of these agents and improve therapy outcomes of patients needing these medications.

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