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

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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 h, with 1.4×10^{-4} M azathioprine and 1.1×10^{-3} M mercaptopurine, concentrations corresponding to the IC_{50} values calculated after 96 h 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 (ANOVA).
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 h and 23.7% and 36.1% after 96 h; 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 h. 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 = 0.0070). 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 and 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.

1. Introduction

The thiopurines azathioprine and mercaptopurine are antimetabolite drugs widely used for their immunosuppressive action.

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These drugs are employed to treat several chronic autoimmune pathologies, to avoid rejection after organ transplantation and as anti-leukemic agents [1]. Thiopurines are prodrugs that require complex conversion (Fig. 1) to be activated to thioguanine nucleotides (TGNs).

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 interesting 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-thiodeoxyGTP (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 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.

2. Materials and method

2.1. 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.

2.2. Treatment with thiopurine drugs

IHH cells (1×10^6) were seeded in 25 cm^2 flasks and treated, for 48 and 96 h, with azathioprine (1.4×10^{-4} M, Sigma-Aldrich, Milan, Italy) and mercaptopurine (1.1×10^{-3} M, Sigma-Aldrich, Milan, Italy) both dissolved 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 h thiopurine exposure previously evaluated in IHH cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

2.3. 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 min at 400×*g*; 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.

2.4. 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.

2.5. 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