# Insights into the cellular pharmacokinetics and pharmacodynamics of thiopurine antimetabolites in a model of human intestinal cells

Elena Genova [a], Marianna Lucafò [a], Marco Pelin [b], Veronica Di Paolo [c], Luigi Quintieri [c], Giuliana Decorti [a,d,*], Gabriele Stocco [b]

[a] Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste, Italy
[b] Department of Life Sciences, University of Trieste, Trieste, Italy
[c] Laboratory of Drug Metabolism, Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy
[d] Department of Medical, Surgical and Health Sciences, University of Trieste, Trieste, Italy

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ABSTRACT

Thiopurines, immunomodulating drugs used in the management of different chronic autoimmune conditions and as anti-leukemic agents, may exert in some cases gastrointestinal toxicity. Moreover, since these agents are administered orally, they are absorbed across the gastrointestinal tract epithelium. On these premises, cellular and molecular events occurring in intestinal cells may be important to understand thiopurine effects. However, quantitative information on the biotransformation of thiopurines in intestinal tissues is still limited. To shed light on biotransformation processes specific of the intestinal tissue, in this study thiopurine metabolites concentrations were analyzed by an *in vitro* model of human healthy colon, the HCEC cell line, upon exposure to cytotoxic concentrations of azathioprine or mercaptopurine; the investigation was carried out using an innovative mass spectrometry method, that allowed the simultaneous quantification of 11 mono-, di-, and triphosphate thionucleotides. Among the 11 metabolites evaluated, TIMP, TGMP, TGDP, TGTP, MeTIMP, MeTIDP and MeTITP were detectable in HCEC cells treated with azathioprine or mercaptopurine, considering two different incubation times before the addition of the drugs (4 and 48 h). Different associations between metabolites concentrations and cytotoxicity were detected. In particular, the cytotoxicity was dependent on the TGMP, TGDP, TGTP and MeTITP concentrations after the 4 h incubation before the addition of thiopurines. This may be an indication that, to study the association between thiopurine metabolite concentrations and the cytotoxicity activity *in vitro*, short growth times before treatment should be used. Moreover, for the first time our findings highlight the strong correlation between cytotoxicity and thiopurine pharmacokinetics in HCEC intestinal cells *in vitro* suggesting that these cells could be a suitable *in vitro* model for studying thiopurine intestinal cytotoxicity.

## 1. Introduction

The thiopurines azathioprine and mercaptopurine are antimetabolite drugs widely used for their immunomodulating action. These drugs are commonly used in the management of different chronic autoimmune conditions, to avoid rejection after organ transplantation and as anti-leukemic agents [1]. Thiopurines are prodrugs that undergo a complex biotransformation (Fig. 1) to exert their activity, carried out by enzymes of the purine salvage pathway [2].

Thioguanine nucleotides (TGNs) represent the active metabolites incorporated into the DNA. This mechanism is the principal cause of cytotoxicity in leukemic blasts and lymphocytes [3]. However, thiopurines may exert cytotoxicity by other mechanisms such as the inhibition of *de novo* purine synthesis, mainly due to methyl-thioinosinic metabolites [4], and of specific signaling pathways in activated lymphocytes [5]. Despite their proven efficacy, thiopurines show severe adverse effects such as pancreatitis, hepatitis and leukopenia in some patients [6]. Interindividual variability in the susceptibility to these adverse effects is related to the complex cellular biotransformation of these agents, involving 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]. Thiopurines have also been associated with gastrointestinal toxicity, especially in patients co-treated with

* Corresponding author. Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste, Italy.
*E-mail address:* decorti@units.it (G. Decorti).

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methotrexate [8]. Among the causes of intestinal toxicity, reduced TPMT activity and consequent metabolites accumulation has been described [9,10]. However, quantitative information on the biotransformation of thiopurines in the intestinal tissues is still limited.

Recently, we measured thiopurine metabolite levels in immortalized human hepatic cells (IHH) using a highly specific and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify simultaneously 11 mono-, di-, and triphosphates thionucleotides [11], instead of the total amount of the phosphorylated forms, as measured by standard methods [12–14]. In this study, we concluded that 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 [11].

The aim of this study is to extend this innovative mass spectrometry assay to the identification and quantification of thiopurine metabolites produced after *in vitro* treatment of human colonic epithelial cells (HCEC) with azathioprine and mercaptopurine, and to associate for the first time the relevant metabolite concentration with the cytotoxic activity of the drugs. Alongside metabolites quantification, we analyzed if different growth times before drug treatment (4 and 48 h) could influence metabolite levels and drug cytotoxicity to test for a potential effect of growth rate at the time of treatment.

## 2. Materials and methods

### 2.1. Cell cultures

The immortalized non-tumor intestinal human epithelial cells derived from human colon biopsies (HCEC) were used [15,16]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM EuroClone, Milan, Italy) low glucose with the addition of 10% fetal bovine serum (Sigma-Aldrich, Milan, Italy), 2 mM L-glutamine (EuroClone, Milan, Italy), 0.3 mM penicillin, 0.02 mM streptomycin (EuroClone, Milan, Italy), 20 mM Hepes buffer (Sigma-Aldrich, Milan, Italy), 1 mM sodium pyruvate (Sigma-Aldrich, Milan, Italy), 0.05 mM ethanolamine (Sigma-Aldrich, Milan, Italy) and $4.9 \times 10^{-4}$ mM O-phosphatidylethanolamine (Sigma-Aldrich, Milan, Italy). This cell line presents a wild type TPMT genotype (Supplementary). 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

HCEC cells ($2 \times 10^6$) were seeded in 25 $cm^2$ flasks and treated for 48 and 96 h, with azathioprine ($7.4 \times 10^{-5}$ M, Sigma-Aldrich, Milan, Italy) and mercaptopurine ($1.8 \times 10^{-4}$ M, Sigma-Aldrich, Milan, Italy) both diluted in NaOH 0.1 M (final concentration of NaOH in the medium, $9.0 \times 10^{-5}$ M). The concentrations of azathioprine and mercaptopurine correspond to the $IC_{50}$ [17] obtained after a 96 h thiopurine exposure previously evaluated in HCEC cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL, Sigma-Aldrich, Milan, Italy) test. Before drug treatment, cells were cultured for 4 or 48 h. Control cultures were treated with the same volume of NaOH 0.1 M used for drug treatment.

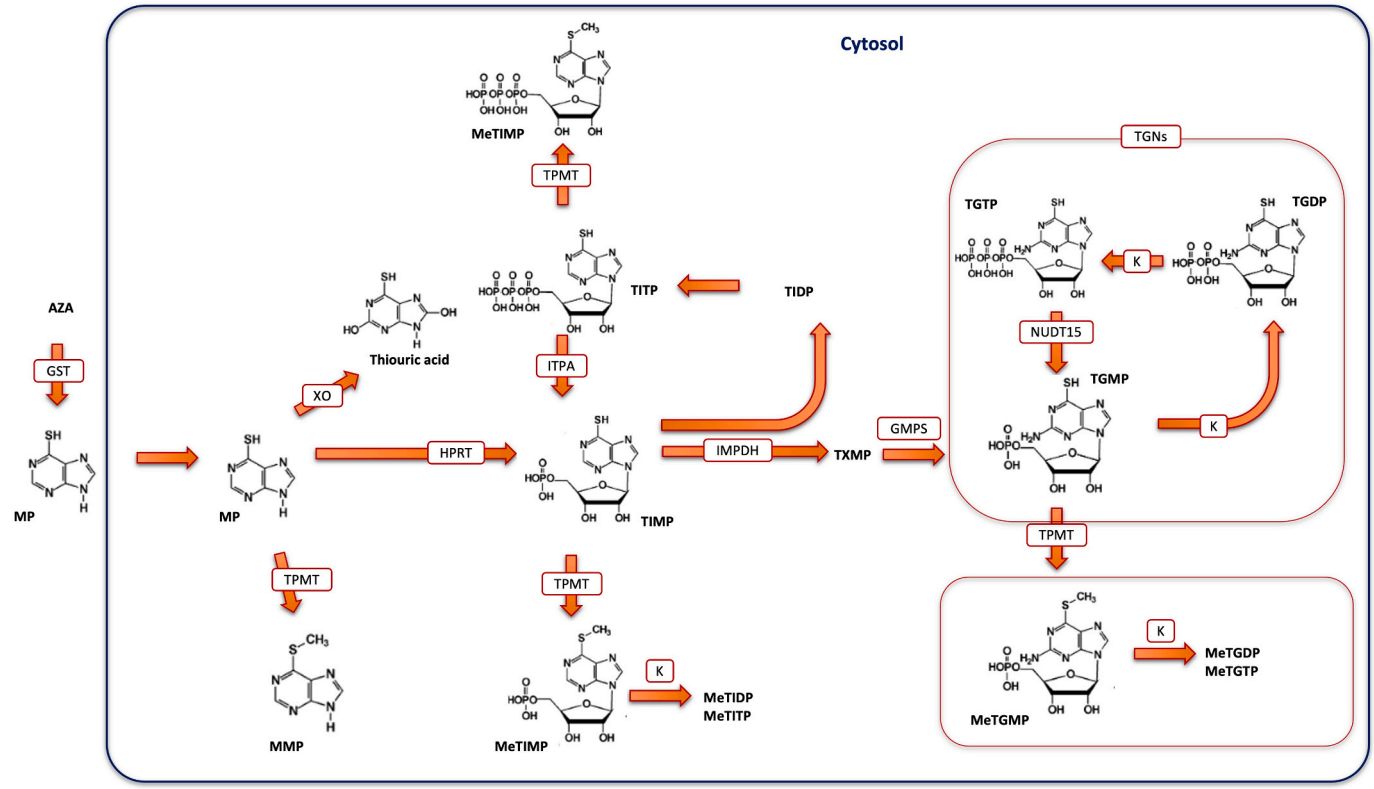


**Fig. 1.** Biotransformation of azathioprine (AZA) and mercaptopurine (MP). MeTGDP, methylthioguanosine 5′-diphosphate; MeTGMP, methylthioguanosine 5′-monophosphate; MeTGTP, methylthioguanosine 5′-triphosphate; MeTIDP, methylthioinosine 5′-diphosphate; MeTIMP, methylthioinosine 5′-monophosphate; MeTITP, methylthioinosine 5′-triphosphate; MMP, methylmercaptopurine; TGDP, thioguanosine 5′-diphosphate; TGMP, thioguanosine 5′-monophosphate; TGTP, thioguanosine 5′-triphosphate; TIDP, thioinosine 5′-diphosphate; TIMP, thioinosine 5′-monophosphate; TITP, thioinosine 5′-triphosphate; Thiouric acid; TXMP, thioxanthine 5′-monophosphate; GMPS, guanosine monophosphate synthetase; GST, glutathione-S-transferase; HPRT, hypoxanthine guanine phosphoribosyltransferase; IMPDH, inosine monophosphate dehydrogenase; ITPA, inosine triphosphate pyrophosphatase, NUDT15, Nudix Hydrolase 15; K, kinase; TPMT, thiopurine S-methyltransferase; XO, xanthine oxidase.

### 2.3. Processing of samples for mass spectrometry assay

After drug treatment, cells were detached using trypsin 0.05% and EDTA 0.02% in PBS (Sigma-Aldrich, Milan, Italy), counted by the Trypan blue dye (Sigma-Aldrich, Milan, Italy) exclusion assay, pelleted by centrifugation for 5 min at 400×*g* and 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 [18]. A mixture of 250 μL of 50 mM EDTA, 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, and 80 pmol/μL [2H4]TGTP/[2H4]TGDP) was added to the cell pellet and vortex mixed. For protein denaturation, samples were heated at 95 °C for 5 min in a water bath, and the subsequent extraction was performed by the addition of 50 μL of methanol followed by the addition of 250 μL of dichloromethane with thorough mixing after each step. After centrifugation at 16,100×*g* for 20 min, 5 μL of the supernatant was used for LC-MS/MS analysis as described previously [18]. Metabolites' quantification was normalized based on the number of viable cells for each sample and was reported as pmol/million 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 dye exclusion assay. Results are reported as viable cell counts in comparison to untreated controls.

### 2.5. Statistical analysis

All data analyses were performed within the R software environment (version 4.0.1) for statistical computing and graphics. Data were analyzed by fitting analysis of variance (ANOVA) models, considering, for the *in vitro* metabolites measurements, each metabolite concentration as the dependent variable and exposure time, time before the addition of the drug and drug used as independent variables, corresponding to an ANOVA with a formula: metabolite concentration ~ exposure time * drug used * time before the addition of the drug. For the *in vitro* cytotoxicity analysis, the percentage of viable cells treated with azathioprine and mercaptopurine in comparison to untreated controls was considered as the dependent variable and metabolites concentrations, exposure time, time before the addition of the drug and drug used as the independent variables, both in univariate ANOVA and in a multivariate analysis combining covariates significant in the univariate analysis. Hypothesizing that the incubation time before the addition of the drug may influence *in vitro* cytotoxic effects of thiopurines, an analysis considering the results of each set of experiments performed with the two times of incubation before the addition of the drug was performed. For each drug and exposure time, experiments were performed in triplicate with the 4 h of incubation before the thiopurine treatment and in duplicate with the 48 h of incubation before the thiopurine treatment.

## 3. Results

### 3.1. HCEC cell counts and treatment with azathioprine and mercaptopurine

In order to assess *in vitro* pharmacokinetics of thiopurine antimetabolites, an *in vitro* study was carried out on HCEC cells, seeding $2 \times 10^6$ cells in 25 $cm^2$ flasks and treating with azathioprine ($7.4 \times 10^{-5}$ M) and mercaptopurine ($1.8 \times 10^{-4}$ M), comparing the two incubation times before drug exposure (4 h vs 48 h). Cells were collected after 48 or 96 h of incubation, counted and processed for thiopurine metabolites quantification by the LC-MS/MS assay.

Considering the percentage of growth of treated cells in comparison to untreated controls, both azathioprine and mercaptopurine induced a significant time-dependent reduction of cell viability (*p*-value ANOVA for treatment duration effect = $2.7 \times 10^{-5}$); no significant differences could be observed between the two drugs or incubation time before drug exposure considering the cytotoxic effect (Fig. 2).

### 3.2. Thiopurine metabolites concentrations in HCEC cells treated with azathioprine and mercaptopurine

For thiopurines metabolites, a summary of the measurements is provided for azathioprine and mercaptopurine in Table 1 (4 h incubation before treatment) and Table 2 (48 h incubation before treatment).

Considering the total amount of all thiopurine nucleotides, there was no change over time (48 h *vs* 96 h) or according to the drug (azathioprine *vs* mercaptopurine) or incubation time before treatment (4 h *vs* 48 h): the mean of the sum of metabolites content was 413.6 pmol/million cells and 382.6 pmol/million cells after 48 and 96 h, respectively.

Considering the relative amount of each metabolite class, the most abundant metabolite class was the methylthioinosine nucleotides, in particular MeTIMP, representing 56.7% of total metabolites after 48 h and 62.1% after 96 h; MeTIDP and MeTITP were also present, even if at a much lower proportion (2.8% for MeTIDP and 2.9% for MeTITP of total metabolites). The other classes of thiopurine nucleotides, ranked according to relative abundance in cells, were thioinosine nucleotides (in particular TIMP, 19.1% of total metabolites) and thioguanine nucleotides (15.8% of total metabolites); methylthioguanine nucleotides were the less abundant, resulting detectable only after 48 h of treatment with azathioprine and with the protocol applying the 48 h incubation time before treatment (0.06% of total metabolites).

Considering the association between each thiopurine metabolite concentration as the dependent variable and exposure time, incubation time before treatment and drug used as independent variables, a significant difference between azathioprine and mercaptopurine could be observed for absolute values of TGMP (*p*-value ANOVA for thiopurine effect = 0.020), with azathioprine having a higher concentration of this metabolite (Fig. 3 A). Both thiopurines used (*p*-value ANOVA for thiopurine effect = 0.00061) and treatment duration (*p*-value ANOVA for treatment duration effect = 0.031) significantly influenced TIMP concentration, which resulted higher after mercaptopurine treatment and reduced over time (Fig. 3 B).

Moreover, for MeTIDP and MeTITP, besides thiopurine used (*p*-value ANOVA for thiopurine effect = 0.048 and 0.032 respectively), with mercaptopurine presenting higher concentrations, also treatment duration (*p*-value ANOVA for treatment duration effect = 0.035 and 0.0051 respectively) and incubation time before treatment (*p*-value ANOVA for incubation time before treatment = 0.0028 and 0.0058 respectively) influenced significantly the metabolite concentration. In particular, increasing concentration of both metabolites over time, using the 48 h incubation time before treatment (Fig. 4) was observed.

Considering the relative amount of each metabolite class and the effect of the thiopurine used, treatment duration and incubation time before treatment, results obtained are similar to those obtained considering the absolute amount of the metabolites (Fig. 5). In particular, for TGMP, the relative amount was higher with azathioprine treatment (*p*-value ANOVA for thiopurine effect = $9.3 \times 10^{-6}$). For TIMP, both thiopurine used (*p*-value ANOVA for thiopurine effect = $8.8 \times 10^{-5}$) and treatment duration (*p*-value ANOVA for treatment duration effect = 0.0058) had a significant effect, with the proportion of this metabolite concentration being higher with mercaptopurine and reduced over time. A difference was detected also considering the relative amount of MeTIMP, MeTIDP and MeTITP, with only incubation time before treatment affecting MeTIMP (*p*-value ANOVA = 0.029). Thiopurine used (*p*-value ANOVA for thiopurine effect = 0.048), treatment duration (*p*-value ANOVA = 0.011) and incubation time before

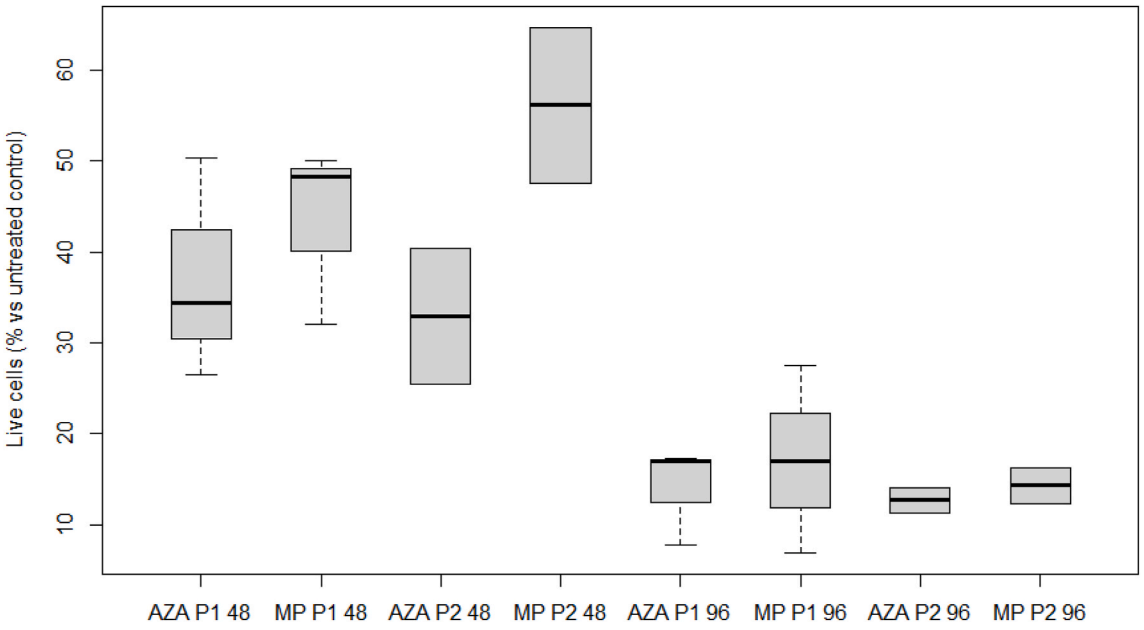


**Fig. 2.** Viable cell counts percentages evaluated by the Trypan blue dye exclusion assay after 48 and 96 h exposure to $7.4 \times 10^{-5}$ M azathioprine (AZA) and $1.8 \times 10^{-4}$ M mercaptopurine (MP). Results are reported as viable cell counts in comparison to untreated controls. P1: 4 h incubation time before treatment, P2: 48 h incubation time before treatment.

**Table 1**
Average concentration of thiopurine metabolites in HCEC cells treated with mercaptopurine and azathioprine for 48 and 96 h with 4 h incubation time before treatment.

| Metabolites | 48 h | | 48 h | | 96 h | | 96 h | |
|---|---|---|---|---|---|---|---|---|
| | Azathioprine | | Mercaptopurine | | Azathioprine | | Mercaptopurine | |
| | 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 | 35.6 ± 20.5 | 17.3% | 169 ± 0.13 | 31.8% | 25.4 ± 8.14 | 9.41% | 64.3 ± 18.1 | 16.7% |
| TGMP | 46.9 ± 13.5 | 22.8% | 34.2 ± 9.10 | 6.43% | 50.5 ± 13.2 | 18.7% | 42.0 ± 10.8 | 10.9% |
| TGDP | 1.11 ± 1.11 | 0.540% | 1.30 ± 0.670 | 0.240% | 2.54 ± 1.77 | 0.94% | 3.40 ± 2.10 | 0.880% |
| TGTP | 0.510 ± 0.510 | 0.250% | 0 | 0% | 1.38 ± 1.38 | 0.510% | 2.22 ± 1.51 | 0.580% |
| MeTIMP | 121 ± 31.9 | 58.7% | 321 ± 69.7 | 60.4% | 178 ± 29.6 | 65.9% | 236 ± 45.73 | 61.2% |
| MeTIDP | 0 | 0% | 2.95 ± 2.95 | 0.550% | 5.02 ± 5.02 | 1.86% | 20.0 ± 7.61 | 5.18% |
| MeTITP | 0.930 ± 0.930 | 0.450% | 3.55 ± 2.14 | 0.670% | 7.37 ± 4.06 | 2.73% | 17.6 ± 5.89 | 4.57% |
| Total thiopurine metabolites | 206 ± 67.2 | 100% | 531 ± 130 | 100% | 270 ± 43.2 | 100% | 38.0 ± 65.0 | 100% |

The analytes TITP, MeTGMP, MeTGDP and MeTITP were not detected. Values are means ± standard errors.

treatment ($p$-value ANOVA = 0.0028) affected MeTIDP, while treatment duration ($p$-value ANOVA = 0.0022) and incubation time before treatment ($p$-value ANOVA = 0.028) affected MeTITP. No other significant difference for the relative amount of thiopurine metabolites was evident over time and according to treatment protocol or thiopurine used (Tables 1 and 2).

### 3.3. Association between thiopurine metabolites and cell viability

Analysis of the association between the cytotoxic effect of the drugs and the concentration of thiopurine metabolites considered the most abundant metabolites, namely MeTIMP, MeTIDP, MeTITP, TIMP, TGMP, TGTP and TGDP, presenting average concentration values above 1 pmol/million cells. A significant association could be detected in the univariate analysis for thiopurines cytotoxic activity and TGDP ($p$-value ANOVA = 0.041), TGTP ($p$-value ANOVA = 0.036), MeTITP ($p$-value ANOVA = 0.022) concentration, with higher metabolite concentrations associated with lower viable cell counts (Fig. 6).

The other metabolites evaluated (MeTIMP, MeTIDP, TGMP) were not significantly associated with a reduction of the number of viable cells, both considering the total and the relative amount. A multivariate analysis considering the association between cell viability reduction and the significant metabolite concentrations, adjusted for treatment duration, confirmed a significant effect of TGMP but not of the other metabolites (Supplementary Table 1) while treatment duration was always significant ($p < 0.0002$).

We hypothesized that incubation times before treatment may affect the mechanism of thiopurines cytotoxicity by influencing the proliferative status of cells at the beginning of treatment. Indeed, a significant association between thiopurine metabolite concentrations and cytotoxicity could be detected only after the 4 h incubation time before treatment (round symbols in Fig. 6), with a significant effect of TGMP, TGDP, TGTP and MeTITP, while after the 48 h incubation time before treatment (triangular symbols in Fig. 6) no significant association was observed for all the metabolites (Table 3).

**Table 2**
Average concentration of thiopurine metabolites in HCEC cells treated with mercaptopurine and azathioprine for 48 and 96 h with 48 h incubation time before treatment.

| Metabolites | 48 h | | 48 h | | 96 h | | 96 h | |
|---|---|---|---|---|---|---|---|---|
| | Azathioprine | | Mercaptopurine | | Azathioprine | | Mercaptopurine | |
| | 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 | 56.0 ± 35.9 | 10.5% | 182 ± 53.0 | 47.5% | 38.6 ± 3.79 | 11.00% | 111.0 ± 42.5 | 21.1% |
| TITP | 0 | 0% | 0 | 0% | 0 | 0% | 0 | 0% |
| TGMP | 84.7 ± 7.12 | 15.9% | 32.8 ± 0.780 | 8.59% | 70.2 ± 12.1 | 20.0% | 40.8 ± 16.5 | 7.79% |
| TGDP | 4.12 ± 0.230 | 0.770% | 3.16 ± 0.085 | 0.820% | 4.86 ± 0.220 | 1.38% | 3.37 ± 0.29 | 0.64% |
| TGTP | 2.75 ± 0.340 | 0.510% | 2.05 ± 0.280 | 0.540% | 3.65 ± 1.13 | 1.04% | 1.78 ± 0.02 | 0.34% |
| MeTIMP | 361.0 ± 20.68 | 67.5% | 127 ± 12.2 | 33.2% | 198 ± 30.7 | 56.4% | 316 ± 41.0 | 60.24% |
| MeTIDP | 14.8 ± 5.32 | 2.77% | 21.3 ± 2.80 | 5.57% | 19.6 ± 3.28 | 5.59% | 22.8 ± 2.72 | 4.35% |
| MeTITP | 10.6 ± 1.84 | 1.98% | 14.4 ± 3.10 | 3.77% | 16.0 ± 0.390 | 4.55% | 29.0 ± 9.04 | 5.54% |
| MeTGMP | 0.340 ± 0.340 | 0.06% | 0 | 0% | 0 | 0% | 0 | 0% |
| MeTGDP | 0.300 ± 0.300 | 0.06% | 0 | 0% | 0 | 0% | 0 | 0% |
| Total thiopurine metabolites | 534 ± 14.8 | 100% | 383 ± 70.5 | 100% | 351 ± 16.3 | 100% | 524 ± 94.0 | 100% |

The analytes TITP and MeTGTP were not detected. Values are means ± standard errors.

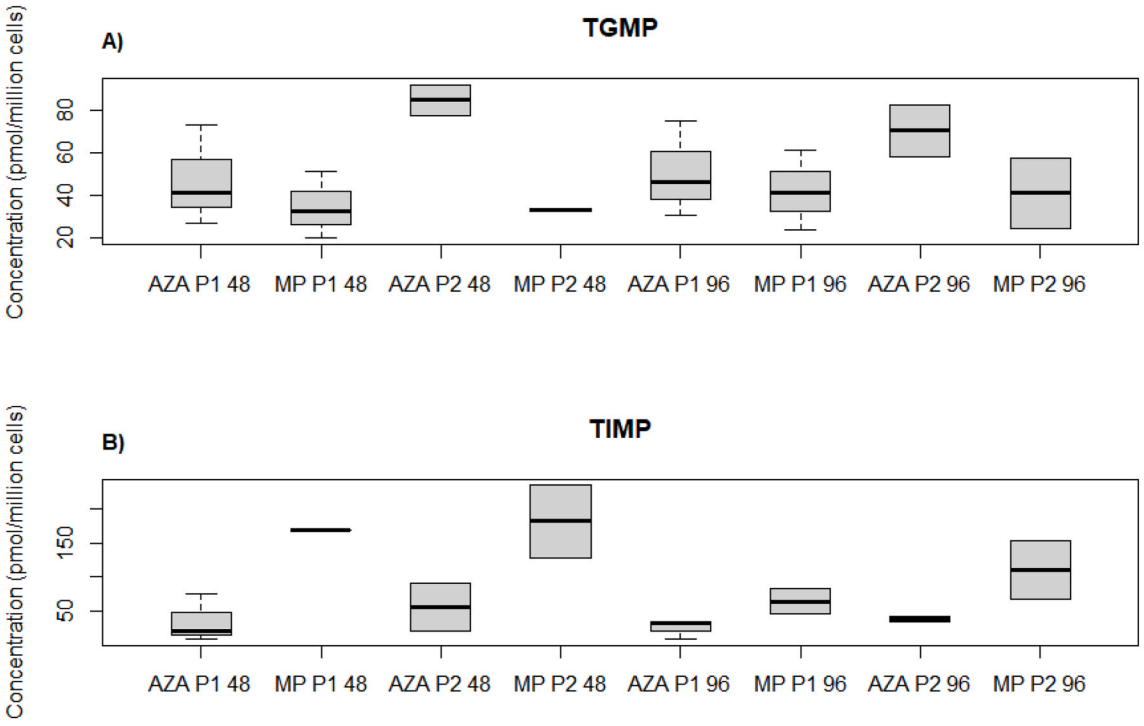


**Fig. 3.** TGMP (panel A) and TIMP (panel B) concentrations in HCEC cells after 48 and 96 h exposure to 7.4 x $10^{-5}$ M azathioprine (AZA) and 1.8 x $10^{-4}$ M mercaptopurine (MP). Results are reported as pmol/million cells. Concentrations of TGMP resulted significantly different according to thiopurine used while concentration of TIMP resulted significantly different according to the thiopurine used and treatment duration (ANOVA). P1: 4 h incubation before the addition of the drugs; P2: 48 h of incubation before the addition of the drugs.

## 4. Discussion

Thiopurines may have specific effects in gastrointestinal cells, since these antimetabolites may exert gastrointestinal toxicity [8], especially when used in patients with acute lymphoblastic leukemia, while in patients with inflammatory bowel disease they typically induce mucosal healing [19]. Moreover, since these agents are administered orally, they are absorbed across the gastrointestinal tract epithelium [20]. On these premises, cellular and molecular events occurring in intestinal cells may be important to understand thiopurine effects. However, quantitative information on the biotransformation of thiopurines in intestinal tissues is still limited. To shed light on biotransformation processes specific of the intestinal tissue, in this study thiopurine metabolites concentrations were analyzed by an *in vitro* model of human healthy colon, the HCEC cell line, upon exposure to cytotoxic concentrations of azathioprine or mercaptopurine; the investigation was carried out using an innovative mass spectrometry method, that allowed the simultaneous quantification of 11 mono-, di-, and triphosphate thionucleotides [18].

In our experimental setting, we applied two different incubation times before treatment, with 4 or 48 h of pre-incubation before drug exposure, in order to test for a potential effect of growth rate at the time of treatment on the cytotoxic activity of thiopurines. Indeed, cytotoxicity of these drugs is strictly related to cell proliferation and cell cycle that can be different after 4 or 48 h of incubation before treatment [21].

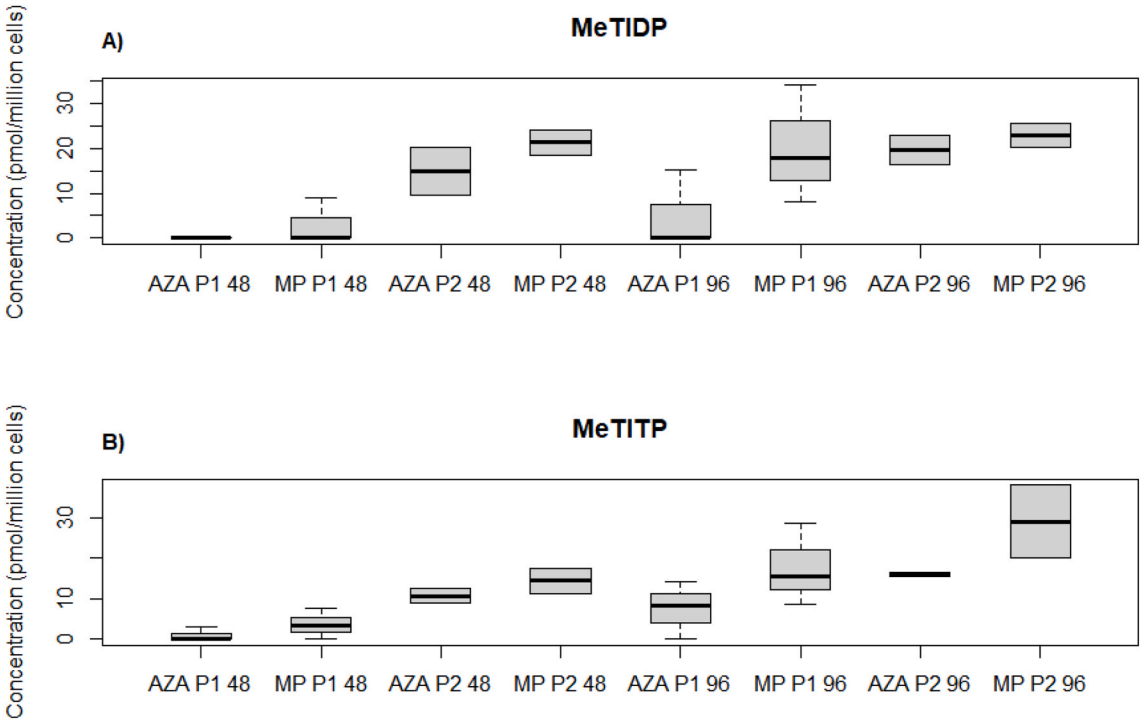


**Fig. 4.** MeTIDP (panel A) and MeTITP (panel B) concentrations in HCEC cells after 48 and 96 h exposure to $7.4 \times 10^{-5}$ M azathioprine (AZA) and $1.8 \times 10^{-4}$ M mercaptopurine (MP). Results are reported as pmol/million cells. Concentrations of MeTIDP and MeTITP resulted significantly different according to thiopurine used, incubation time before treatment and treatment duration. P1: 4 h incubation before the addition of the drugs; P2: 48 h of incubation before the addition of the drugs.

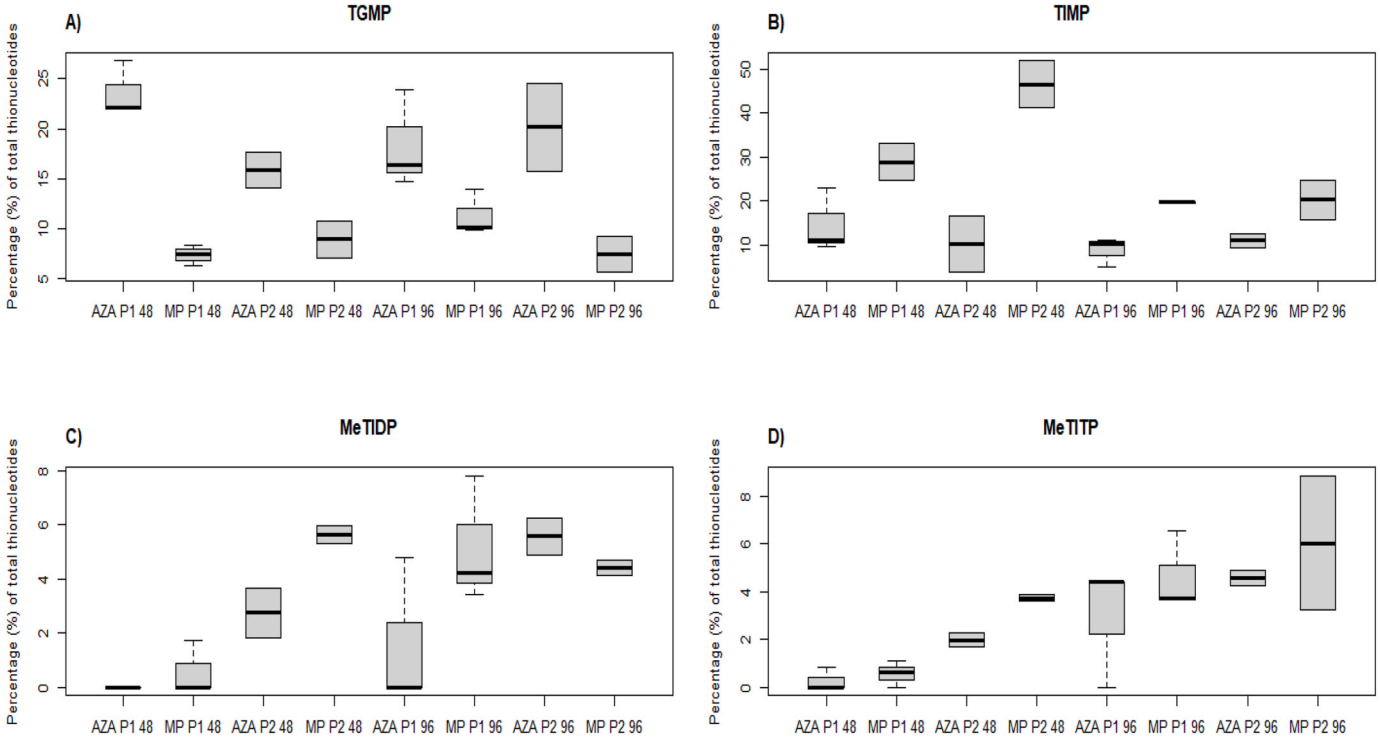


**Fig. 5.** Relative amount of thionucleotides significantly affected by thiopurine used (azatioprine, AZA or mercaptopurine, MP), treatment duration or incubation time before treatment. Results are reported as the percentage of the significant metabolite on the total amount of thionucleotides. Relative amount of TGMP (panel A) was significantly different according to thiopurine used; relative amount of TIMP (panel B) was significantly different according to both thiopurine used and treatment duration, of MeTIDP (panel C) according to all three covariates and of MeTITP (panel D) according to treatment duration and incubation time before treatment. P1: 4 h incubation before the addition of the drugs; P2: 48 h of incubation before the addition of the drugs.

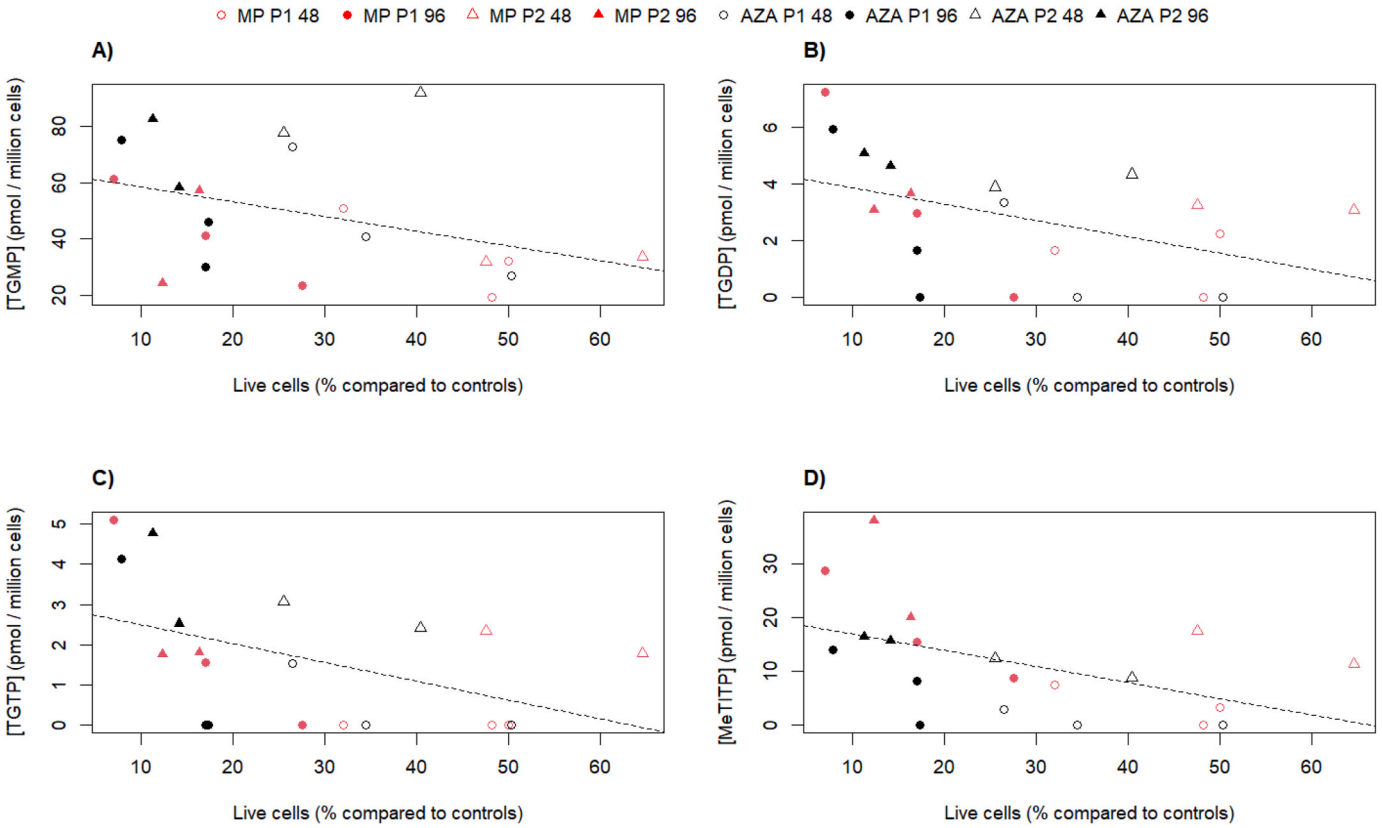


**Fig. 6.** Association analysis between thiopurines concentrations in HCEC cells and thiopurines induced cytotoxicity (% of live cell counts of treated cells with respect to untreated controls, determined by Trypan blue dye assay). Figures contain combined data for azathioprine (AZA) and mercaptopurine (MP) experiments and for the two incubation times before treatment and treatment durations (48 and 96 hours). P1: 4 h incubation before the addition of the drugs; P2: 48 h of incubation before the addition of the drugs.

**Table 3**
Association (regression coefficient and ANOVA *p*-value) between the metabolites concentration and cytotoxicity according to the incubation times before treatment protocol applied.

| | All samples | 4 h incubation before treatment | 48 h incubation time before treatment |
|---|---|---|---|
| TGMP | **−0.40, 0.082** | **−0.63, 0.029** | −0.25, 0.54 |
| TGDP | **−0.46, 0.041** | **−0.64, 0.023** | −0.50, 0.21 |
| TGTP | **−0.47, 0.036** | **−0.68, 0.014** | −0.37, 0.36 |
| TIMP | 0.39, 0.11 | 0.33, 0.34 | 0.45, 0.26 |
| MeTIMP | −0.22, 0.35 | −0.098, 0.76 | −0.40, 0.33 |
| MeTIDP | −0,32, 0.17 | −0.56, 0.060 | −0.21, 0.62 |
| MeTITP | **−0.51, 0.022** | **−0.71, 0.0099** | −0.50, 0.21 |

Among the 11 metabolites evaluated, TIMP, TGMP, TGDP, TGTP, MeTIMP, MeTIDP and MeTITP were detectable in HCEC cells treated with azathioprine or mercaptopurine considering both incubation times before treatment. Interestingly, no significant differences were highlighted in terms of cytotoxicity and total metabolite concentrations between the two incubation times before treatment. However, we found a different association between metabolite concentrations and cytotoxicity. In particular, the cytotoxicity was dependent on the TGMP, TGDP, TGTP and MeTITP levels after the 4 h pre-incubation. This may be an indication that, to study the association between thiopurine metabolite concentrations and the cytotoxicity activity *in vitro*, short pre-incubation times should be used. The 48 h incubation before treatment caused a higher concentration of MeTIDP and MeTITP, which may be responsible for cytotoxicity of cells by inhibiting *de novo* purine synthesis [22]. On the other hand, after a 4 h incubation before treatment, cell cultures may have a higher proportion of cells in DNA synthesis phase, which may be more sensitive to thioguanine nucleotides [23,24].

In our HCEC intestinal model, the levels of thioguanine nucleotides TGMP, TGDP, and TGTP were correlated to cytotoxicity. This correlation is in accordance with literature, where it is reported that these metabolites exert cytotoxic activity by their incorporation in DNA [25]. Alongside thioguanine nucleotides, also the methylated form of thioinosine metabolite MeTITP correlated with cell survival. However, no particular evidence of MeTITP and cytotoxicity correlation are reported in the literature, where only the mono-phosphate form MeTIMP was strictly correlated with cell survival by inhibiting the *de novo* purine synthesis, resulting in strong cytotoxic effects [13,25]. On the other hand, triphosphate nucleotides are considered more cytotoxic than monophosphates [25].

Considering the differences in metabolite concentrations among the thiopurines used, a significant difference between azathioprine and mercaptopurine was found for TIMP, MeTIDP, MeTITP and TGMP, with thioinosine derivatives resulting around 20% higher after mercaptopurine treatment. This may be due to the fact that each thiopurine was used at an equitoxic concentration ($EC_{50}$) that, however, was higher for mercaptopurine than azathioprine ($1.8 \times 10^{-4}$ M mercaptopurine *vs* $7.4 \times 10^{-5}$ M azathioprine), or to the fact that azathioprine needs conversion to mercaptopurine, by reaction with nucleophiles such as reduced glutathione [7,26]. TGMP levels were about 2.5 times higher after azathioprine than that after mercaptopurine treatment. This could be due to the inhibition of the conversion of TIMP to TGMP by IMPDH caused by higher TIMP concentrations after mercaptopurine treatment. Indeed, TIMP is a time-dependent inactivator of IMPDH [27,28].

Interestingly, the cytotoxic activities of the two drugs in our *in vitro* system were comparable; therefore, in our model, the differences highlighted compensate each other.

Overall, considering the total absolute amount of thiopurine metabolites, there was no change over time (48 vs 96 h) or according to thiopurine (azathioprine *vs* mercaptopurine) or seeding protocol (4 h *vs* 48 h of incubation before treatment).

In comparison to HCEC, the correlation between cytotoxicity and thiopurine metabolites levels, that we previously analyzed in hepatic IHH cells with 4 h growth time before thiopurine exposure for 48 and 96 h [11], resulted limited to MeTIMP, while in the intestinal cell line four metabolites were associated with the cytotoxic activity. These findings highlight the strong correlation between cytotoxicity and thiopurine pharmacokinetics in HCEC intestinal cells *in vitro*. Anyway, in intestinal cells, only TGMP was associated with cytotoxicity after adjustment for treatment duration. Expression of *NUDT15* gene, encoding for a nucleoside diphosphate hydrolase, has been reported higher in intestinal cells than hepatic cells [29] and this could be associated with a higher concentration of TGMP. However, increased expression of *NUDT15* seems inconsistent with the greater cytotoxic potency of TGMP compared with TGDP and TGTP.

Interestingly, in our previous study based on metabolite quantification in IHH hepatic cells [11], a decrease in total metabolite amount between 48 and 96 h of treatment was detected. These differences could be due to a distinctive metabolite processing fate in the two cell lines, with a faster biotransformation in IHH cells. Indeed, the liver is the principal site of thiopurine metabolism with a sophisticated detoxification system [30]. The efflux transporters ATP binding cassette subfamily C member 4 (ABCC4) and ABCC5 have been found to be associated with the efflux of thiopurine-derived nucleotides, a process critical for cellular detoxification. However, the expression of these transporters in hepatocytes and colonic cells is comparable [31–33]. Furthermore, the thiopurine influx transporters solute carrier (SLC) 19A1 and SLC19A2 are also equally expressed in colon and liver tissues [34,35].

However, the downregulation of other influx transporters gene expression such as SLC28A2 (CNT2), SLC28A3 (CNT3), SLC29A1 (ENT1) and SLC29A2 (ENT2) has been shown to make lymphocyte-derived cell-lines resistant to thiopurines because of a reduced uptake of thiopurines in cells [36]. In our case, these genes are reported [37–40] to be more expressed in colonic cells, suggesting a higher thiopurine influx. This could explain the almost overall higher total metabolite concentrations found in the present study in colonic cells, in comparison to those previously measured in hepatocytes (Supplementary Table 2). Besides thiopurine transporters, HPRT is a fundamental enzyme in thiopurine activation and its expression and activity can affect metabolite concentration [41]. However, in our case, IHH and HCEC lines seem to have a similar HPRT expression [42] suggesting no influence by this enzyme in terms of metabolite concentration.

Aldehyde oxidase (AO) and xanthine oxidase (XO) contribute to mercaptopurine detoxification through the production of hydroxythioguanine and thiouric acid, respectively [43]. In particular, being AO metabolism highly predominant in hepatocytes [44], we hypothesize that the higher AO activity in hepatocytes could be partially responsible for metabolites reduction after 96 h of thiopurine treatment in IHH cells, in comparison to the constant metabolite levels in HCEC cells. Probably, also other mechanisms that still need to be clarified are involved in thiopurine-derived nucleotides efflux and detoxification.

## 5. Conclusions

Results obtained in this work point out for the first time a significant correlation between thiopurine cytotoxicity and thiopurine metabolites levels in HCEC cells, a human intestinal cell model. Thiopurines are known to induce, in some cases, gastrointestinal toxicity [45]. Therefore, the HCEC intestinal cell line can be a good model for studying thiopurine intestinal toxicity.

## CRediT authorship contribution statement

**Elena Genova:** Investigation, Methodology, Software, Data curation, Writing – original draft, Writing – review & editing. **Marianna Lucafò:** Data curation, Writing – review & editing. **Marco Pelin:** Data curation, Writing – review & editing, Investigation. **Veronica Di Paolo:** Investigation, Writing – review & editing. **Luigi Quintieri:** Supervision, Writing – review & editing. **Giuliana Decorti:** Writing – original draft, Writing – review & editing, Validation. **Gabriele Stocco:** Conceptualization, Supervision, Software, Data curation, Writing – original draft, Validation, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2021.109624.

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