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Research paper

SERS spectroscopy as a tool for the study of thiopurine drug pharmacokinetics in a model of human B leukemia cells

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ABSTRACT

Thiopurine drugs are immunomodulatory antimetabolites relevant for pediatric patients characterized by dose-dependent adverse effects such as myelosuppression and hepatotoxicity, often related to inter-individual differences, involving the activity of important enzymes at the basis of their biotransformation, such as thiopurine S-methyltransferase (TPMT).

Surface Enhanced Raman Scattering (SERS) spectroscopy is emerging as a bioanalytical tool and represents a valid alternative in terms of affordable costs, shorter analysis time and easier sample preparation in comparison to the most employed methods for pharmacokinetic analysis of drugs.

The aim of this study is to investigate mercaptopurine and thioguanine pharmacokinetics by SERS in cell lysates of a B-lymphoblastoid cell line (NALM-6), that did (TPMT*1) or did not (MOCK) overexpress the wild-type form of TPMT as an *in vitro* cellular lymphocyte model to discriminate between cells with different levels of TPMT activity on the base of the amount of thioguanosine nucleotides (TGN) metabolites formed.

SERS analysis of the cell lysates was carried out using SERS substrates constituted by Ag nanoparticles deposited on paper and parallel samples were used for quantification of thiopurine nucleotides with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

A direct SERS detection method has been set up that could be a tool to study thiopurine drug pharmacokinetics in *in vitro* cellular models to qualitatively discriminate between cells that do and do not overexpress the TPMT enzyme, as an alternative to other more laborious techniques.

Results underlined decreased levels of TGN and increased levels of methylated metabolites when TPMT was overexpressed, both after mercaptopurine and thioguanine treatments.

A strong positive correlation (Spearman's rank correlation coefficient rho = 0.96) exists between absolute quantification of TGMP (pmol/1 x 10^6 cells), obtained by LC-MS/MS, and SERS signal (intensity of TGN at 915 cm^{-1}).

In future studies, we aim to apply this method to investigate TPMT activity in pediatric patients' leukocytes.

1. Introduction

The thiopurine drugs mercaptopurine and thioguanine are antimetabolites used for the treatment of pediatric acute lymphoblastic leukemia, autoimmune disorders such as inflammatory bowel disease (IBD) and to prevent rejection after organ transplantation [1]. Thiopurines are inactive prodrugs that undergo a biotransformation through

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Abbreviations

$AgNO_3$	silver nitrate
Ag NPs	silver nanoparticles
CI	confidence interval
C_{int}	intermediate concentration
EC50	half maximal effective concentration
FBS	fetal bovine serum
HPRT	hypoxanthine guanine phosphoribosyl-transferase
IBD	inflammatory bowel disease
ITPA	inosine triphosphate pyrophosphatase
K	kinase
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MeMP	methylmercaptopurine
MeTG	methylthioguanine
MeTGDP	methylthioguanosine 5′-diphosphate
MeTGMP	methylthioguanosine 5′-monophosphate
MeTGTP	methylthioguanosine 5′-triphosphate
MeTIDP	methylthioinosine 5′-diphosphate
MeTIMP	methylthioinosine 5′-monophosphate
MeTITP	methylthioinosine 5′-triphosphate
MP	mercaptopurine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NaOH	sodium hydroxide
SERS	Surface Enhanced Raman Scattering
SNP	single nucleotide polymorphism
TG	thioguanine
TGMP	thioguanosine 5′-monophosphate
TGDP	thioguanosine 5′-diphosphate
TGTP	thioguanosine 5′-triphosphate
TIMP	thioinosine 5′-monophosphate
TITP	thioinosine 5′-triphosphate
TPMT	thiopurine-S-methyltransferase

complex metabolic processes (Fig. 1). Mercaptopurine is first converted into thioinosine 5′-monophosphate (TIMP) by the enzyme hypoxanthine guanine phosphoribosyl-transferase (HPRT). Through two consecutive enzymatic steps, TIMP is then converted to thioguanine nucleotides (TGN) metabolites which are responsible for the cytotoxicity of thiopurines because of their incorporation into nucleic acids [2]. Thioguanine is biotranformed directly to TGN via HPRT. Another metabolic pathway consists in the conversion of thiopurines by the cytosolic enzyme thiopurine-S-methyltransferase (TPMT) into the inactive methylated bases methylmercaptopurine (MeMP) and methylthioguanine (MeTG). MeMP and MeTG have no antileukemic activity since are not converted into nucleotides, as they are poor HPRT substrates [3]. Thioinosine metabolites (i.e. TIMP, TIDP, TITP) and TGN can also be substrates of TPMT and be converted respectively into methylmercaptopurine ribonucleotides (i.e. MeTIMP, MeTIDP, MeTITP) and methylthioguanine nucleotides (i.e. MeTGMP, MeTGDP, MeTGTP) [4,5].

Efficacy of thiopurines is well established; however, thiopurines are characterized by a narrow therapeutic window and dose-dependent adverse drug reactions such as bone marrow toxicity, gastrointestinal toxicity and hepatitis [1]. The susceptibility to these adverse effects is often related to inter-individual differences in the activity of important enzymes involved in thiopurine metabolism and in the purine salvage pathway. It is now well recognized that TGN levels are influenced by TPMT activity: reduced TPMT activity results in accumulation of active metabolites leading to myelosuppression and intestinal toxicity, while in presence of high TPMT activity TGN levels may not be sufficient for therapeutic efficacy [6]. TPMT enzymatic activity has been recognized

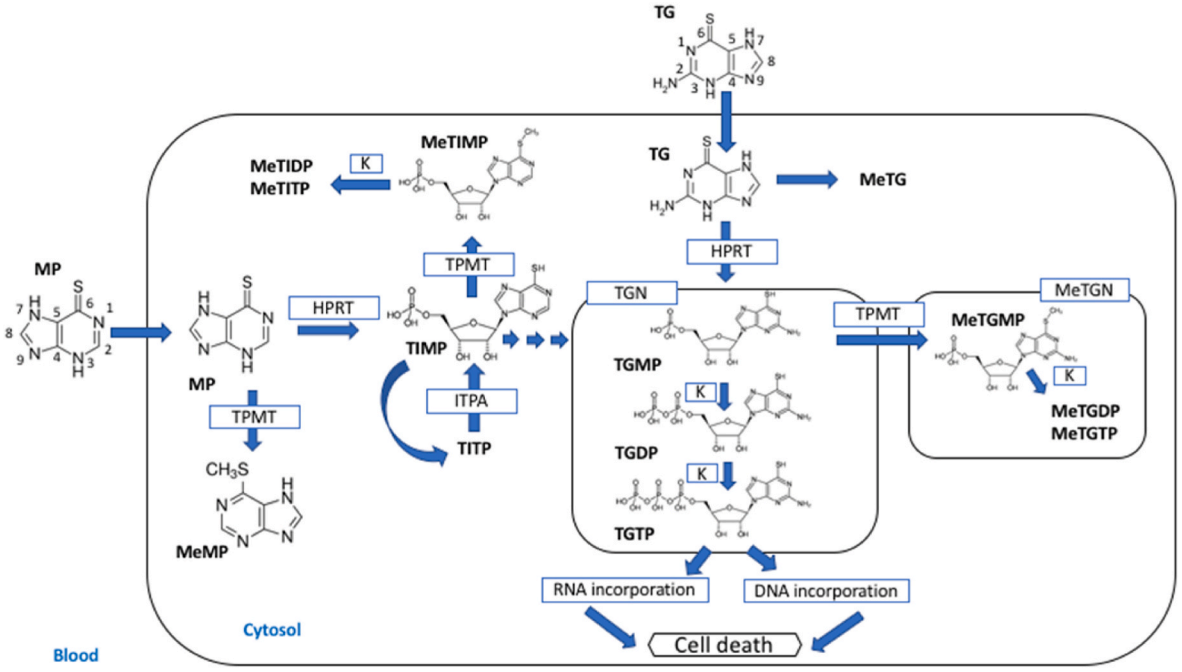

Fig. 1. Biotransformation of mercaptopurine (MP) and thioguanine (TG). HPRT, hypoxanthine guanine phosphoribosyl-transferase; ITPA, inosine triphosphate pyrophosphatase; K, kinase; MeTGDP, methylthioguanosine 5′-diphosphate; MeTGMP, methylthioguanosine 5′- monophosphate; MeTGTP, methylthioguanosine 5′-triphosphate; MeTIDP, methylthioinosine 5′-diphosphate; MeTIMP, methylthioinosine 5′-monophosphate; MeTITP, methylthioinosine 5′-triphosphate; MeMP, methylmercaptopurine; MeTG, methylthioguanine; TGMP, thioguanosine 5′-monophosphate; TGDP, thioguanosine 5′-diphosphate; TGTP, thioguanosine 5′-triphosphate; TIMP, thioinosine 5′-monophosphate; TITP, thioinosine 5′-triphosphate; TPMT, thiopurine S-methyltransferase.

as a monogenic trait [7]. Many studies have shown that there are more than 38 allelic variants responsible for a possible alteration of TPMT activity [8]. However, 95% of the variants causing lower or absent TPMT function comprise only few alleles [9]. *TPMT*2* (rs1800462) is defined by the 238G > C transversion, *TPMT*3* family alleles are defined by the SNPs 460G > A (rs1800460) and 719A > G (rs1142345), in particular *TPMT*3A* is characterized by both 460G > A and 719A > G transitions, *TPMT*3B* corresponds to the SNP rs1800460 and *TPMT*3C* to the SNP rs1142345 [8]. Among these variants, *TPMT*3A* is the most frequent in the Europeans. The most frequent variant in South-east Asian and African populations is *TPMT*3C* [10]. In Africans there is also an additional polymorphism, the 644 G>A transition (rs56161402, *TPMT*8)*, that is putatively presenting reduced enzymatic activity [11]. However, besides the *TPMT* defective variants, other factors such as concomitant drug administration, other pathologies or epigenetic factors can contribute to modify TPMT enzyme activity [12,13].

The use of an assay detecting *in vivo* TPMT activity may discover more patients at risk, providing complementary information to genetic analysis and a higher precision in the treatment personalization, since genetic variants are not the only cause of altered TPMT activity in patients [8].

Nowadays liquid chromatography-tandem mass spectrometry (LC-MS/MS) is probably one of the most practical approach for drug and metabolite quantification. However, it is clear that LC-MS/MS together with the other most employed methods for pharmacokinetic analysis of drugs such as high-pressure liquid chromatography and fluorescence spectroscopy requires expensive and time-consuming sample processing and analysis in comparison to Surface Enhanced Raman Scattering (SERS) spectroscopy.

SERS is emerging in the biomedical field as a bioanalytical tool, and represents a valid alternative owing to its intrinsic features i.e. high sensitivity and capability to recognize fingerprints. Thanks to the technological advancements the Raman instruments provided are more cost-effective and user-friendly transforming it to a more widely available analytical technique. Moreover SERS requires short analysis time and easy sample preparation [14].

SERS spectra are representative of the vibrational fingerprint of the molecule analyzed. The technique is based on the amplification of the Raman signal emitted by the molecule of interest interacting with an electromagnetic radiation and adsorbed onto nanoparticles of noble metals (mostly silver, gold or copper) able to considerably increase the local magnetic field. Concerning the determination of enzyme activity by SERS, several studies have shown its feasibility by investigating the influences of the enzyme reaction on the substrate molecule. Stevenson R. et al. described the first intracellular Raman enzyme detection method, employing metallic nanoparticles to selectively detect a molecule that presents Raman spectra only after interaction with the target enzyme [15]. A SERS based method to investigate protease activity consisting in monitoring cleavage of peptide substrates was reported by Turk N. and colleagues [16]. In this work, we evaluated a direct SERS detection method to monitor the influence of the TPMT activity level on the pharmacokinetics of thiopurine drugs in cell lysates of a B-lymphoblastoid cell line (NALM6) that did or did not overexpress the wild-type form of TPMT, on the base of the amount of TGN formed. NALM6 cell line was chosen as an *in vitro* model of human leukocytes which are the main target cells of thiopurine drugs.

The active thiopurine metabolite levels and their methylated forms produced after *in vitro* treatment with mercaptopurine and thioguanine were also measured using LC-MS/MS and the results were compared to those obtained by SERS.

2. Materials and methods

2.1. Chemicals and reagents

Sodium citrate tribasic dihydrate (C7254) and silver nitrate (AgNO3, 204390), sodium chloride (NaCl, S9888), fetal bovine serum (FBS, F7524-500 ML), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT, M2128), mercaptopurine (852678), thioguanine (A4882), trypan blue solution (T8154), Tris base (T1503), Tween-20 (P7949), DMSO (472301-2.5L), phosphate buffered saline (PBS, 806552-500 ML), Amicon Ultra 0.5 mL centrifugal filters with 10 kDa cut-off (UFC501096), Gene Elute Blood Genomic DNA Kit (NA2010), Goat Anti-Rabbit IgG Antibody, Peroxidase Conjugated (AP132P) were purchased from Sigma-Aldrich (Milan, Italy). Sodium hydroxide (NaOH, 106498) was purchased from Merck (Milan, Italy). RPMI 1640 (ECB9006L), L-glutamine (ECB3000D), penicillin/streptomycin (ECB3001D), LiteAblo TURBO Extra Sensitive Chemiluminescent Substrate (EMP012001) were obtained from EuroClone (Milan, Italy). TGMP (NU-1121L), TGDP (NU-1120L), TGTP (NU-1106L) were purchased from Jena Bioscience (Jena, Germany). Milli-Q water was used for cleaning procedures and for preparation of solutions. Filter paper (qualitative filter paper, 410, with 2 μm pore size, 28321) was purchased from VWR International (Milan, Italy).

TaqMan™ SNP Genotyping Assay (Assay ID: C_12091552_30, C_30634116_20; C_19567_20), Power Blotter Pre-cut membranes and filters nitrocellulose regular size (PB7320), NuPAGE 10% Bis-Tris Gel 12-well (NP0302BOX) were obtained from ThermoFisher (Milan, Italy). Anti-TPMT antibody E-8 (sc-374154) was purchased from Santa Cruz Biotechnology (Dallas, USA). Anti-mouse IgG, HPR-linked Antibody (#7076) was purchased from Cell Signaling technology (Danvers, USA). Recombinant Anti-Actin antibody (ab218787) was obtained from Abcam (Cambridge, UK).

2.2. Cell culture

The human B-lymphoblastoid cell line NALM6 with forced expression of TPMT (NALM6 TPMT*1) and relative control (NALM6 MOCK) were kindly provided by Prof. William Evans (St. Jude Children's Research Hospital, Memphis, USA), and generated as described in Stocco et al., 2012 [17].

NALM6 cells were grown in suspension at 37 °C and 5% CO_2 in standard T25 flasks (833910002, Sarstedt, Numbrecht, Germany) and were seeded at a concentration of 1 x 10^6 cells/ml at every cellular passage for maintenance that was performed every 3–4 days. The medium used for the culture of these cells was RPMI 1640 supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 10% FBS.

2.3. Cytotoxicity assay

NALM6 MOCK and NALM6 TPMT*1 cells (20×10^3 cells/well) were exposed for 72 h to mercaptopurine (3×10^{-7} M to 2×10^{-5} M) and thioguanine (1×10^{-7} M to 1×10^{-4} M). Mercaptopurine and thioguanine solutions were both prepared in NaOH 0.1 M. In the last 4 h of treatment, a solution of MTT was added (final concentration 0.5 mg/mL) and the crystals were solubilized with 100 μL of DMSO. The absorbance was read by an automated microplate reader (FLUOstar Omega, BMG Labtech, Germany) at 540/630 nm. Cells sensitivity was calculated as the half maximal effective concentration (EC50) at 72 h from the dose-response curves.

2.4. DNA extraction and TaqMan SNP genotyping assay

Total genomic DNA was isolated from NALM6 MOCK and NALM6 TPMT*1 pellets (5 x 10^6 cells) using a commercial kit (Gene Elute Blood Genomic DNA Kit), according to the manufacturer's protocol.TaqMan SNP genotyping assays were used to characterize the single nucleotide polymorphisms (SNPs) of interest: TPMT rs1800462 (238G > C), rs1800460 (460G > A) and rs1142345 (719A > G) Samples genotyping was done in a technical triplicate.

2.5. Western Blotting assay

To verify the stability of the transfection with plasmid encoding for the wild type form of TPMT, NALM6 MOCK and TPMT*1 were collected and lysed to extract the respective protein lysates. Whole cell extracts (15 μg) were fractionated by SDS-PAGE using NuPAGE 10% Bis-Tris Gel 12-well and transferred to nitrocellulose membranes using Electrophoresis Power Supply (EPS301, Thermo Fischer Scientific, Italy). After incubation with 5% nonfat milk (non-phospho-protein) in Tris buffered saline (50 mM Tris base, 25 mM NaCl, pH 7.5) with 0.1% Tween 20 (T-TBS) for 1 h, the membranes were incubated with antibodies against: TPMT (1:500 in T-TBS 5% nonfat milk) and actin (1:3000 in T-TBS 5% nonfat milk) at 4 °C, overnight. Membrane incubated with actin antibody was washed and incubated for 1 h at 4 °C with a 1:20000 dilution in T-TBS 5% nonfat milk of peroxidase-conjugated anti-rabbit antibodies, while the membrane incubated with TPMT antibody was incubated for 1 h at 4 °C with a 1:3000 dilution in T-TBS 5% nonfat milk of anti-mouse antibodies. Blots were developed with LiteAblo TURBO Extra Sensitive Chemiluminescent Substrate following the manufacturer's instructions.

2.6. Cell treatment with thiopurines and cell lysate preparation for thiopurine metabolites quantification by LC-MS/MS and SERS analysis

The EC50 at 72 h and the intermediate concentration (C_{int}) for mercaptopurine and thioguanine, calculated as the mathematical mean between the EC50 at 72 h for NALM6 MOCK and TPMT*1, were used for cell treatments. Controls were treated with NaOH 0.1 M at the same volume used for drug treatment, since both mercaptopurine and thioguanine were prepared in NaOH 0.1 M. Four biological replicates were done both for mercaptopurine and thioguanine treatments. After drug treatment for 24 and 48 h, cell suspension was collected, counted by the trypan blue dye exclusion assay, centrifuged for 5 min at 400×*g* and the pellet was washed with 10 mL of sterile PBS to eliminate any medium left-over. For each condition, two dry pellets were prepared and stored at −80 °C until analysis. One pellet (>2 x 10^6 cells) was used for the quantification of thiopurine metabolites by LC-MS/MS [18]. Metabolite quantification was normalized on the number of viable cells and expressed as pmol/1x10^6 cells.

The other pellet (2.5 x 10^6 cells) was analyzed by SERS at the Department of Engineering and Architecture of the University of Trieste (Italy). For SERS analysis, sodium citrate solution (10 mM, pH 4.5, 200 μL) was added to the cell pellet (2.5 x 10^6 cells). Cells were sonicated using a sonicator (Hielscher UP50H) set to 1 cycle and 80 of vibrational amplitude, the lysis was carried out on ice for 15–20 s. Subsequently the lysate was placed in filtering devices (Amicon Ultra 0.5 mL) with a cut-off equal to 10 KDa and centrifuged for 30 min at 14000 g to separate the larger size cellular components of the filtrate to prevent interference in SERS spectra. Until use, the filtered lysate was maintained at −80 °C.

2.7. SERS substrates preparation and characterization

Solid SERS substrates were obtained by coating paper with silver nanoparticles (Ag NPs). Ag NPs synthesis was conducted according to a protocol described by Lee-Meisel [19]. A solution of $AgNO_3$ (90 mg dissolved in 500 mL of Milli-Q water)was heated to boiling. Under vigorous magnetic stirring, sodium citrate tribasic solution 1% (10 mL) was added drop-by-drop. The boiling solution was kept under heat and stirring for 60 min. The colloidal solution of Ag NPs was stored in the dark at room temperature. The colloids characterization was performed by UV–Vis absorption spectroscopy for each batch using a Lambda 20 bio UV–Vis spectrometer (Perkin-Elmer, Monza, Italy). The surface plasmon band of the metal colloid provides information about the average dimension and about the dispersion of the NPs, whose variability affects the Raman enhancement. The Ag NPs surface plasmon band was detected between 403 and 411 nm and was consistent with the values previously reported in literature [20]. The solid SERS substrates were then obtained using a method described by Wu-Li-Ji Hasi [21], which consists in coating the filter paper with Ag NPs. A 2.0 × 5.0 cm rectangle of filter paper (qualitative filter paper, 410, with 2 μm pore size) was cut and was left completely immersed in 20 ml of NaCl solution (0.3 M in Milli-Q water) for 20 min, allowing the dispersion of the chloride ions along the entire surface of the paper. The filter paper was then air-dried at room temperature, and cut into 1 cm^2 pieces. Each piece was placed at the bottom of a well in a 24-multiwell plate (832932, Sarstedt, Numbrecht, Germany) then a colloidal solution of Ag NPs (1.5 mL) was added in each well and the plate was stored in the dark at room temperature for 24 h. After this time, the supernatant was removed with a syringe, avoiding touching the substrate. The substrates were dried under a ventilated hood overnight and then stored in Milli-Q water and kept in the dark until use.

2.8. SERS analysis

SERS spectra were measured at room temperature with an i-RamanPlus, a portable Raman spectrometer equipped with a microscope with PL 20X objective lens (B&W Tek, Newark, USA). The excitation source consisted in a high-power laser (500 mW) with an emission at 785 nm. The Raman spectrometer was equipped with a detector with a wide spectral coverage (65-4200 cm^{-1}) and a spectral resolution of 4.5 cm^{-1} (@912 nm). Before each measurement, the calibration of the instrument was checked using as reference the bands of monocrystalline silicon (single band at 520 cm^{-1}) and of paracetamol (multiple bands).

Drops of 5 μL of each analyte were deposited on the solid silver substrates and allowed to dry for about 15 min before SERS measurements. All spectra of treated cell lysates were recorded using an accumulation of 3 scans (10 s exposure) at 5 random locations on the surface of 5 different substrates, with 10 mW of laser power focused onto the sample. Spectra of untreated cell lysates (controls) were recorded at 4 random locations on the surface of 3 different substrates, using the same instrument settings. The optimization of instrument settings was determined to maximize signal, avoid saturation and sample degradation deriving from laser excitation. The software used to collect data was BWSpec version 4.03_23_c (B&W Tek, Newark, USA).

Comparison of LC-MS/MS and SERS spectroscopy analysis features is displayed in Table 1.

2.9. Data pre-processing, analysis and visualization

In order to quantify the protein bands from western blot, the relative amounts as a ratio of each protein band relative to the β-actin as loading control were calculated with ImageJ software. Data are presented as mean fold change ± SEM (n = 3).

The EC50 of mercaptopurine and thioguanine calculated after 72 h of exposure and the C_{int} between the EC50 at 72 h of NALM6 MOCK and TPMT*1 were calculated with the software Prism9 – GraphPad.

All spectral data were preprocessed and plotted using the R language (version 4.1.0) [22] with RStudio (version 1.4.1106) [23]. Spectra were

Table 1
Comparison of LC-MS/MS and SERS spectroscopy analysis features.

Features	LC-MS/MS analysis	SERS spectroscopy analysis
Sample needed for analysis	5 μL	5 μL
Time for sample preparation	2 h	About 30 min
Time for analysis	15.50 min per sample	10 s per sample
Number of samples assayed	Illimited (about 3–4 samples per hour)	Illimited (about 360 samples per hour)
Versatility of monitoring parent and metabolites	Versatile	Versatile
Specificity of assay	Specific	Thioguanine nucleotides are collectively detected

exported from the instrument as ASCII data (.txt) and imported into the R environment as a single hyperSpec object using the hyperSpec package [24], attaching metadata by extracting them from filenames. Median spectra for each biological replicate were calculated from 5 technical replicates from 5 individual substrates (i.e. 25 different measurements of the same biological replicate).

Median spectra were then cropped to the 400-1800 cm^{-1} Raman shifts range. A baseline (package baseline [25], method = "als", lambda = 4) was subtracted from each spectrum. Intensity was normalized (vector normalization) for data used in Fig. 3. Band positions were automatically detected and then labelled using the detectPeaks and labelPeaks functions of the MALDIquant package [26]. For the Gardner-Altman plots, CI were estimated with bias-corrected and accelerated nonparametric bootstrap resampling using the DABEST (Data Analysis with Bootstrap Estimation) package [27]. This enables visualization of the confidence interval without making any assumption about the distributions.

3. Results

3.1. Cell line characterization

Genotypes of the three most common variants of *TPMT* (rs1800462 (238G > C), rs1800460 (460G > A) and rs1142345 (719A > G)) was investigated in NALM6 MOCK and NALM6 TPMT*1 and resulted to be wild-type for the three SNPs (data not shown). The protein expression of TPMT was assessed by Western blotting analysis, using anti-TPMT antibodies on cellular extracts (Fig. 2).

The amount of protein expression for total TPMT (exogenous at 38 kDa and endogenous at 32 kDa), in terms of average fold change to TPMT in NALM6 MOCK, was equal to 3.6 ± 0.7 (mean ± SEM, n = 3) for NALM6 TPMT*1. The effects of the overexpression of the TPMT enzyme in NALM6 TPMT*1 on the response to mercaptopurine and thioguanine (72 h of treatment), evaluated by the MTT test, were compared with the results obtained for NALM6 MOCK. The overexpression of TPMT in NALM6 resulted in an increase in sensitivity for mercaptopurine (Fig. 3A), significantly decreasing the EC50 (equal to 0.53 x 10^{-6} M; 95% CI: 0.47 x 10^{-6} -0.57 x 10^{-6} M) of almost 3 times ($p < 0.0001$, two-way ANOVA) compared to NALM6 MOCK (EC50 equal to 1.44 x 10^{-6} M; 95% CI 1.1 x 10^{-6} -1.81 x 10^{-6} M). On the contrary, the increased expression of TPMT decreases sensitivity for thioguanine at 72 h of treatment. In particular, the overexpression increases the EC50 (Fig. 3B) (equal to 7.36 x 10^{-6} M; 95% CI 6.03 x 10^{-6} - 8.98 x 10^{-6} M) by about 3 times ($p < 0.0001$, two-way ANOVA) compared to NALM6 MOCK (EC50 equal to 2.45 x 10^{-6} M; 95% CI 1.93 x 10^{-6} –3.11 x 10^{-6} M). The C_{int} of mercaptopurine was calculated as 1 μM, while the C_{int} of thioguanine was calculated as 5 μM.

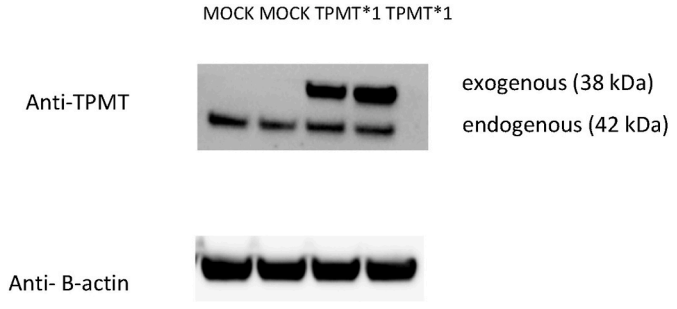

Fig. 2. Western Blotting assay images for protein expression of TPMT (exogenous at 38 kDa and endogenous at 32 kDa) in cellular extracts of NALM6 MOCK and NALM6 TPMT*1 (two replicates for each cell line). Protein expression of B-actin (42 kDa) was used as loading control.

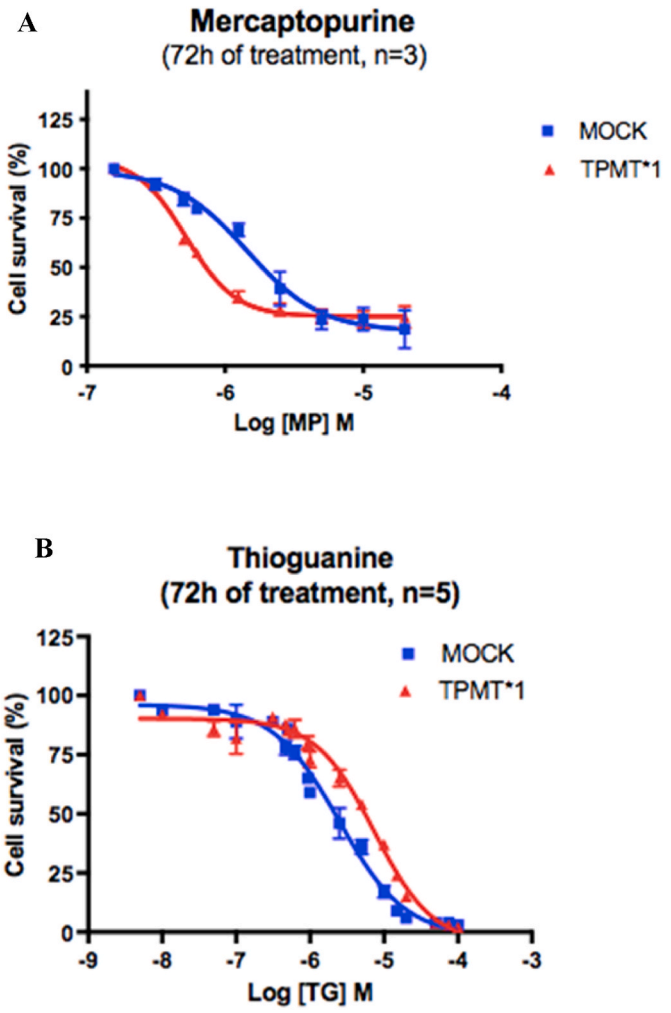

Fig. 3. Dose-response curves of mercaptopurine (A) and thioguanine (B) for NALM6 MOCK (in blue) and NALM6 TPMT*1 (in red) cells after 72 h of drug treatment, n ≥ 3. Data shown in Fig. 3A and B are the means ± SEM of at least three independent MTT assay experiments and are reported as % of cell survival (absorbance treated/absorbance untreated control × 100).

3.2. SERS analysis of thiopurine metabolites in cell lysates of NALM6 MOCK and TPMT*1 treated with mercaptopurine and thioguanine

SERS spectra of mercaptopurine, thioguanine and TGN solutions are reported in Fig. 4A and B, together with the spectra of the lysates of treated NALM6 MOCK cells. The marked differences between SERS spectra of these molecules, due to their different chemical structures, clearly allow to distinguish between each parental drug and TGN, their active metabolites. The metabolites collectively referred to as TGN (i.e. TGMP, TGDP and TGTP) have identical SERS spectra, since they only differ by the number of phosphates, while the moiety involved in the interaction with the Ag is the same, though they present different intensities of the characteristic bands (Fig. 5). Moreover, spectra in Fig. 4A and in Fig. 4B also show that only TGN spectral patterns are detected in the lysates of treated cells (at 24 h after treatment), suggesting that parental drugs have been mostly metabolized. The absence of parental drugs' spectral features is more evident for MP than for TG, as the former has a SERS spectrum presenting more differences in comparison to TGN. However, at a closer inspection the same conclusion can be drawn for TG: although both TG and TGN have an intense band at 915 cm^{-1}, the presence of TG in the lysates can be excluded from the absence of any band around 1300 cm^{-1}, where SERS spectra of this drug features its most intense band. In other words, it can be safely concluded that the

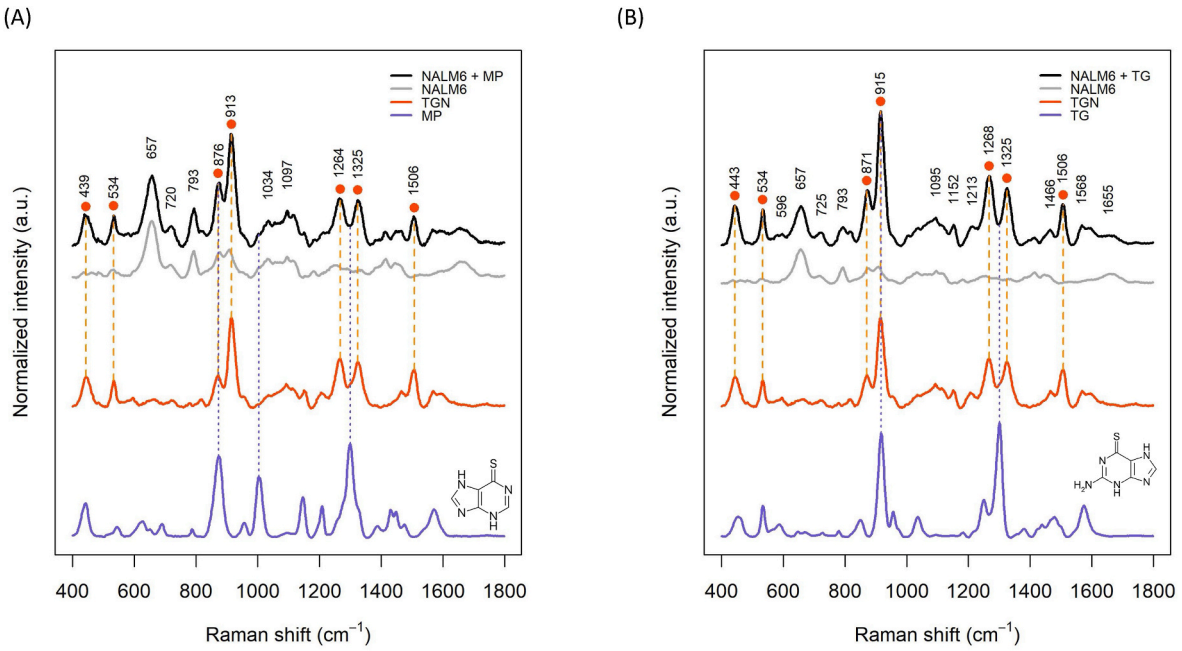

Fig. 4. Average SERS spectra of NALM6 MOCK cell lysates treated with mercaptopurine (1 μM) **(A)** or thioguanine (5 μM) for 24 h **(B)** in black, average SERS spectra of cell lysates of untreated NALM6 MOCK in grey, average SERS spectra of solutions of pure TGN (mean of TGMP, TGDP, TGTP at 10^{-5} M) in orange, mercaptopurine (10^{-6} M) or thioguanine (10^{-5} M) in violet. Red dots indicate TGN more intense peaks identified in the treated cell lysates.

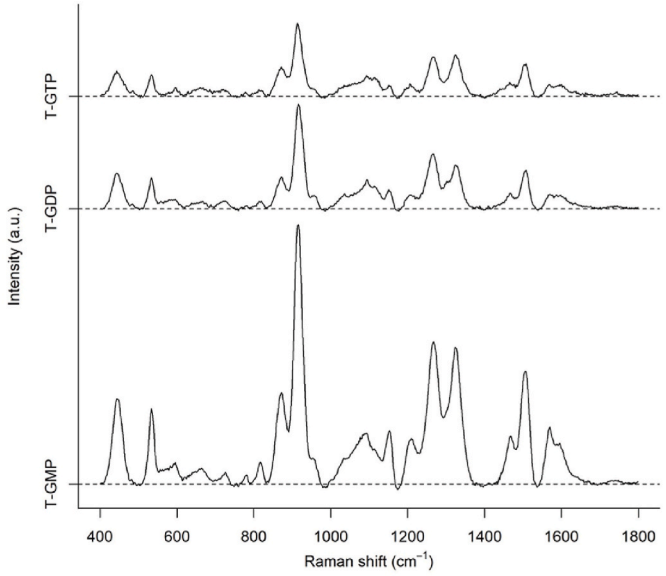

Fig. 5. Average SERS spectra of pure TGMP, TGDP and TGTP at 10^{-5} M in buffer sodium citrate 10 mM, pH 4.5.

SERS signal at 915 cm^{-1} in the SERS spectra of lysates cells treated with TG is not due to the parental drug but to TGN.

Figs. 6 and 7 show the superimposed SERS spectra of NALM6 MOCK (in blue) and NALM6 TPMT*1 (in red) cell lysates after treatment for 24 and 48 h with mercaptopurine (EC50 and C_{int}) and thioguanine (EC50 and C_{int}), respectively. The difference spectrum is displayed (in black) at the bottom of each chart.

The Gardner-Altman plots [27], in Figs. 6 and 7, present the band intensity at 915 cm^{-1} (i.e. the Raman shift of the most intense TGN band) for NALM6 MOCK and NALM6 TPMT*1 cell lysates in all biological replicates, and the difference in medians, indicated by the black dot with its 95% confidence interval (CI).

After 24 h of exposure to mercaptopurine (EC50 and C_{int}), a difference is evident in terms of lower levels of TGN formed in NALM6 TPMT*1, especially after the treatment with EC50 concentration (Fig. 6C). With the increased incubation time to 48 h, the SERS signal of TGN is still appreciable even if reduced both in NALM6 MOCK and TPMT*1 and the difference is less significant (Fig. 6B and D). These results suggest that TGN were further biologically converted over time or incorporated in the synthesis of DNA/RNA.

The Gardner-Altman plots show the observed values (intensity of the peak at 915 cm^{-1} of NALM6 MOCK and TPMT*1 cell lysates) in all biological replicates (n = 4), highlighting the two groups' comparison by a difference axis that displays the effect size, here the difference of medians indicated by the black circle. The median of the test group (NALM6 MOCK) is the difference-axis origin (zero). The difference of the medians is flanked by the confidence interval (CI) which is illustrated by the vertical line.

Contrary to what has been observed for treatment with mercaptopurine, after 24 h of exposure to thioguanine (EC50 and C_{int}), the intensities of the band at 915 cm^{-1} of TGN in NALM6 MOCK and TPMT*1 are comparable (Fig. 7A and C). After 48 h, the cytosolic levels of TGN are instead significantly lower in NALM6 TPMT*1 than in NALM6 MOCK, especially with C_{int} of thioguanine treatment (Fig. 7B).

*3.3. Mass-spectrometry analysis of NALM6 MOCK and TPMT*1 treated with mercaptopurine and thioguanine: quantification of thiopurine metabolites*

Concentration of thiopurine metabolites analyzed by means of LC-MS/MS assay are reported in Table 2 (treatment with drug concentration corresponding to EC50) and Table 3 (treatment with the C_{int}, 1 μM mercaptopurine and 5 μM thioguanine). Figs. 8 and 9 show the Gardner-Altman plots for the concentration of TGN (pmol/1 x 10^6 cells) obtained by mass-spectrometry analysis in NALM6 MOCK and TPMT*1, after treatment with mercaptopurine and thioguanine (EC50 and C_{int}) for 24 and 48 h in all biological replicates.

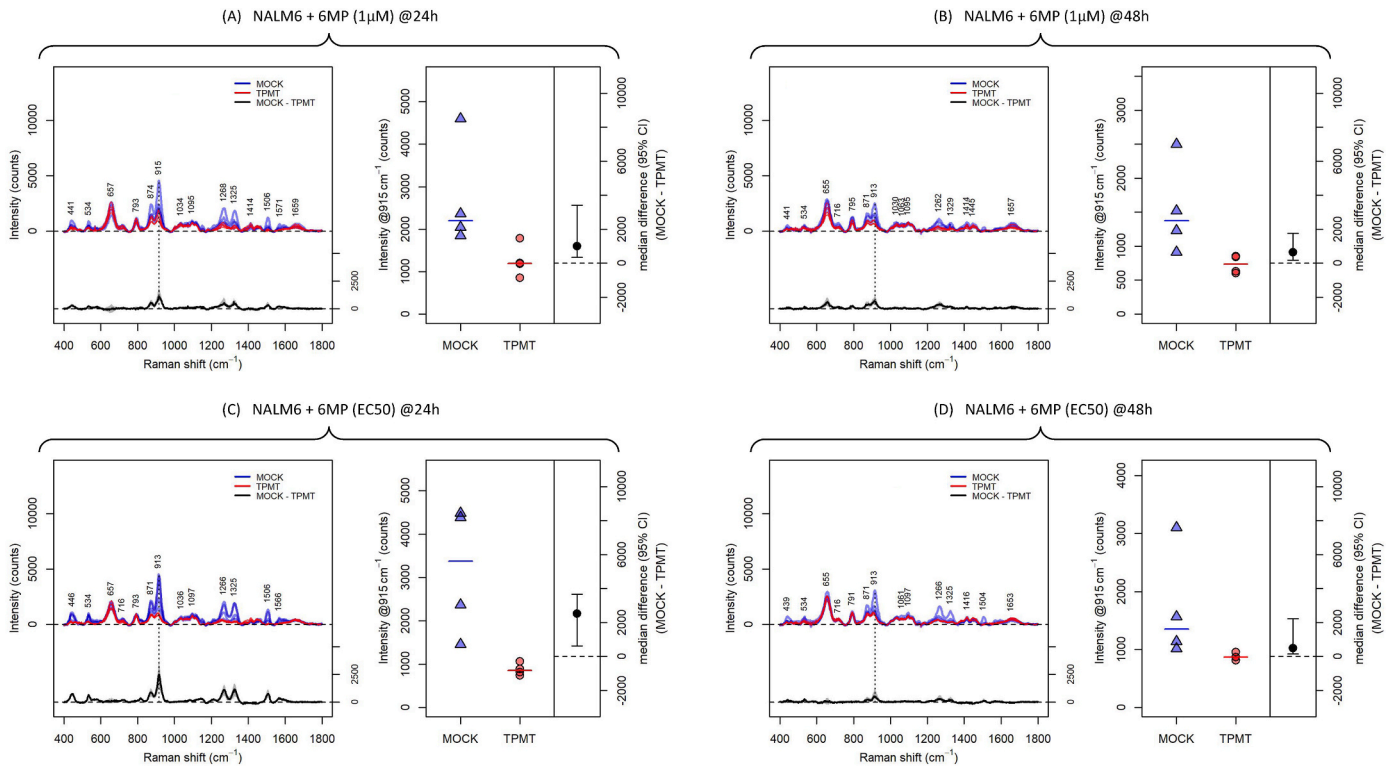

Fig. 6. SERS spectra of NALM6 MOCK (in blue) and NALM6 TPMT*1 (in red) cell lysates after treatment with mercaptopurine (C_{int} and EC50) for 24 (Fig. 6A and C) and 48 h (Fig. 6B and D). The difference spectrum is displayed (in black) at the bottom of each chart. The matched Gardner-Altman plots show the observed values (intensity of the peak at 915 cm^{-1} of NALM6 MOCK and TPMT*1 cell lysates) in all biological replicates (n = 4), highlighting the two groups' comparison by a difference axis on the right that displays the effect size, here the difference of medians indicated by the black circle. The median of the test group (NALM6 MOCK) is the difference-axis origin (zero). The difference of the medians is flanked by the confidence interval (CI) which is illustrated by the vertical line.

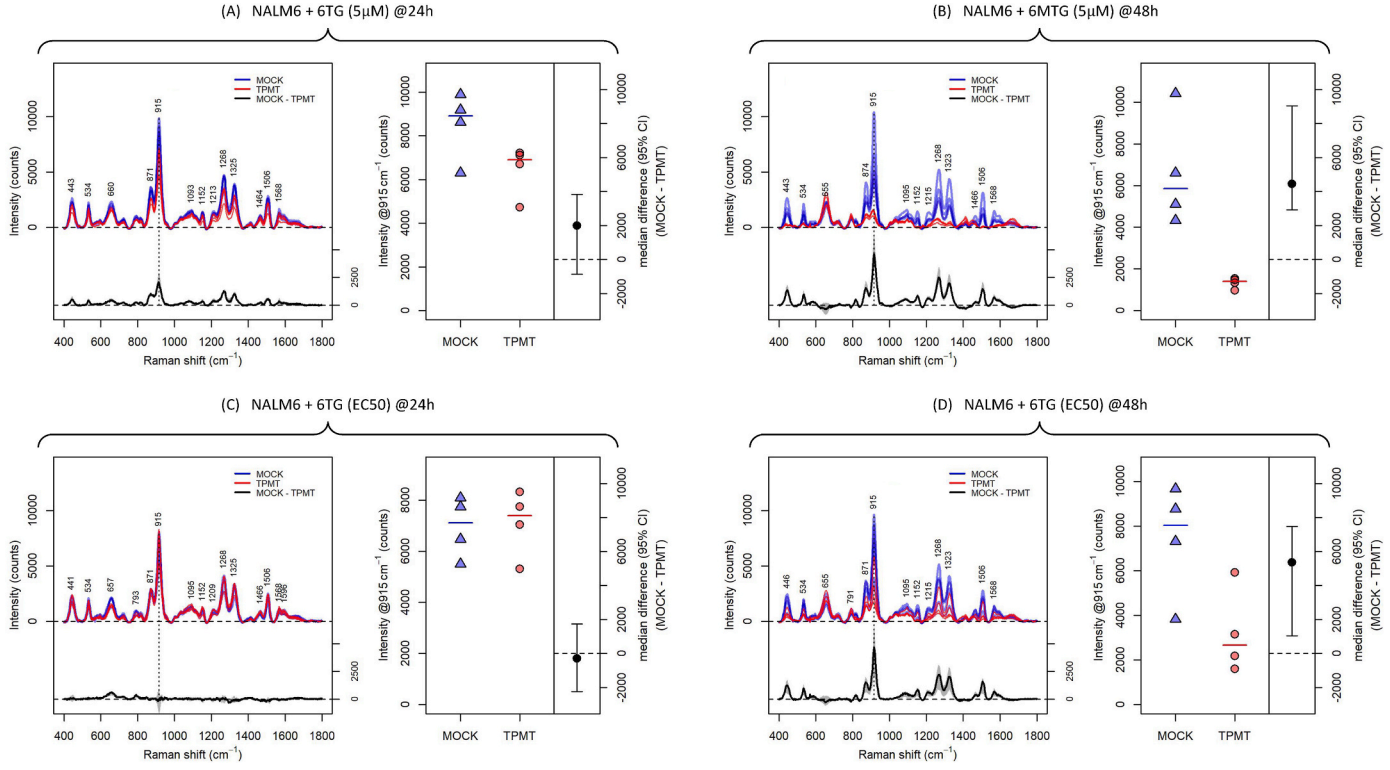

Fig. 7. SERS spectra of NALM6 MOCK (in blue) and NALM6 TPMT*1 (in red) cell lysates after treatment with thioguanine (C_{int} and EC50) for 24 (Fig. 7A and C) and 48 h (Fig. 7B and D). The difference spectrum is displayed (in black) at the bottom of each chart with the corresponding interquartile distributions represented by the shaded area.

Table 2
Average concentration of thiopurine metabolites in NALM6 MOCK and TPMT*1 cell lysates treated with EC50 concentration of mercaptopurine and thioguanine for 24 and 48 h, measured by mass-spectrometry. Values are expressed as means (n = 4) ± standard errors.

Metabolites	24 h		24h		48h		48h	
	mercaptopurine		thioguanine		mercaptopurine		thioguanine	
	MOCK	TPMT*1	MOCK	TPMT*1	MOCK	TPMT*1	MOCK	TPMT*1
	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells
TIMP	9.8 ± 3.7	0	0	0	0	0	0	0
TIDP	0	0	0	0	0	0	0	0
TITP	0	0	0	0	0.2 ± 0.2	0	0	0
TGMP	106.4 ± 32.1	11.6 ± 3.4	406.9 ± 30.9	378.4 ± 32.8	50.3 ± 24.0	0.4 ± 0.2	492.8 ± 42.8	203.6 ± 88.1
TGDP	1.8 ± 1.0	0	35.5 ± 1.4	26.8 ± 2.7	0.6 ± 0.6	0	40.0 ± 3.0	9.4 ± 5.0
TGTP	6.9 ± 2.3	0	132.5 ± 12.3	92.5 ± 10.0	2.2 ± 1.7	0	130.7 ± 9.9	32.1 ± 18.0
MeTIMP	41.1 ± 13.0	117.4 ± 44.6	0	0	36.5 ± 9.0	105.3 ± 32.7	0	0
MeTIDP	0	0	0	0	0	2.4 ± 2.1	0	0
MeTITP	0	1.2 ± 1.1	0	0	0	2.1 ± 1.9	0	0
MeTGMP	4.8 ± 1.8	4.8 ± 1.5	22.3 ± 1.2	124.2 ± 11.5	3.4 ± 1.7	0	30.5 ± 4.1	116.1 ± 39.8
MeTGDP	0.6 ± 0.3	0.4 ± 0.2	11.9 ± 0.9	52.3 ± 5.8	0.6 ± 0.4	0	14.4 ± 1.5	41.9 ± 16.5
MeTGTP	0.5 ± 0.3	0.3 ± 0.2	19.3 ± 2.4	74.4 ± 4.4	0.4 ± 0.4	0	14.7 ± 5.2	52.1 ± 19.4

Table 3
Average concentration of thiopurine metabolites in NALM6 MOCK and TPMT*1 cell lysates treated with the C_{int} 1 μM mercaptopurine and 5 μM thioguanine for 24 and 48 h, measured by mass-spectrometry. Values are expressed as means (n = 4) ± standard errors.

Metabolites	24 h		24h		48h		48h	
	mercaptopurine		thioguanine		mercaptopurine		thioguanine	
	MOCK	TPMT*1	MOCK	TPMT*1	MOCK	TPMT*1	MOCK	TPMT*1
	pmol/1 x $10^{6.}$ cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells	pmol/1 x 10^6 cells
TIMP	6.9 ± 2.3	0	0	0	0	0	0	0
TIDP	0	0	0	0	0	0	0	0
TITP	0	0	0	0	0	0	0	0
TGMP	93.8 ± 27.3	19.2 ± 7.0	440.5 ± 26.1	426.6 ± 37.6	41.8 ± 17.9	1.2 ± 0.5	374.0 ± 3.3	27.8 ± 4.0
TGDP	2.1 ± 0.8	0	36.7 ± 2.1	27.7 ± 2.6	0	0	32.0 ± 0.6	0
TGTP	5.8 ± 1.9	0.4 ± 0.4	152.0 ± 4.4	92.1 ± 13.9	1.4 ± 1.1	0	114.2 ± 6.8	0.9 ± 0.5
MeTIMP	31.9 ± 12.0	154.8 ± 40.5	0	0	34.5 ± 8.8	165.7 ± 49.0	0	0
MeTIDP	0	0	0	0	0	3.4 ± 1.3	0	0
MeTITP	0	1.5 ± 0.9	0	0	0	5.6 ± 2.0	0	0
MeTGMP	4.2 ± 1.5	7.6 ± 2.7	32.5 ± 2.0	131.5 ± 6.7	2.6 ± 0.9	0	24.2 ± 1.9	21.7 ± 2.8
MeTGDP	0.3 ± 0.3	1.2 ± 0.4	11.8 ± 1.0	54.8 ± 2.0	0.4 ± 0.3	0	11.2 ± 0.2	3.9 ± 0.7
MeTGTP	0.1 ± 0.1	0.9 ± 0.3	21.3 ± 1.6	79.3 ± 9.7	0.3 ± 0.3	0	15.8 ± 1.5	4.4 ± 1.0

4. Discussion

TPMT is a ubiquitous cytosolic enzyme responsible for the formation of methylated thiopurines metabolites and its activity is also related to TGN cytoplasmic concentration [28]. Low TPMT activity is associated to an increased risk of developing severe adverse effects to thiopurine treatment. In particular, patients with low TPMT activity have higher risk of encountering hematologic toxicity, due to the accumulation of TGN [29]. On the contrary, it has not been demonstrated that high TPMT activity could lead to treatment resistance due to subtherapeutic TGN levels, while studies have suggested it could lead to hepatotoxicity due to high MeMP ribonucleotides concentrations [30]. Before starting therapy with thiopurines, drug regulatory agencies such as Food and Drug Administration (FDA) recommended to evaluate TPMT genetic status or activity level, while the American College of Gastroenterology indicates phenotyping as the first choice for patients suffering from IBD with the purpose to identify individuals with altered enzymatic activity and optimize dosage to increase efficacy and tolerance [31,32]. Pharmacogenetic based clinical guidelines are also available [32,33]. However, TPMT activity level is not always fully explained by TPMT defective variants, but other factors such as concomitant drug administration, other pathologies or epigenetic factors can contribute to modify enzyme activity [12,13].

Given the clinical and the experimental evidences accumulated over years and demonstrating a different metabolism of thiopurines and treatment response based on different TPMT activity level [34], this study wanted to assess whether SERS is able to investigate the influence of TPMT activity on the *in vitro* metabolism of thiopurine drugs mercaptopurine and thioguanine, with the intention to discriminate TGN levels between cells, possibly aiming at a future translation of the SERS method to patient samples treated with thiopurines, as an alternative to genetic TPMT analysis. Specifically, we used a B-lymphoblastoid cell line (NALM6) overexpressing the wild-type form of TPMT gene (TPMT*1) compared to NALM6 with the empty vector as control (MOCK).

As reported in other *in vitro* studies [35], cells expressing high TPMT were more sensitive to the cytotoxic effects of mercaptopurine and less sensitive to those of thioguanine accordingly to our MTT assay results. These opposite contribution of TPMT to the two drugs have already been explained suggesting that over physiological methylation of mercaptopurine *in vitro* contributes to the antiproliferative properties of the drug, probably through inhibition of de novo purine synthesis by methyl mercaptopurine nucleotides, whereas thioguanine is inactivated primarily by TPMT with a decrease of TGN levels [335]. These hypotheses are also supported by our mass-spectrometry analysis for the quantification of thiopurine metabolites, that show higher levels of MeTGN in NALM6 TPMT*1 after treatment with mercaptopurine and a reduction of TGN in NALM6 TPMT*1 after treatment with thioguanine.

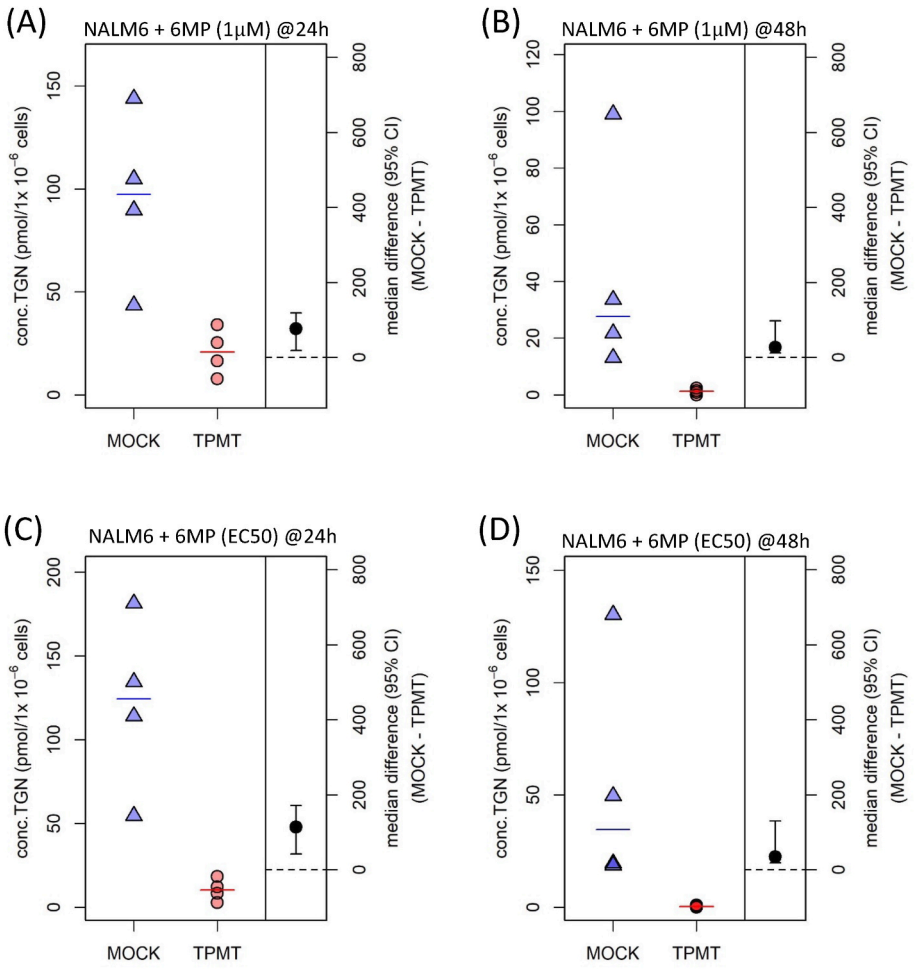

Fig. 8. Gardner-Altman plots of the concentration of all TGN (pmol/1 x 10^6 cells) measured by LC-MS/MS in NALM6 MOCK and TPMT*1 treated with mercaptopurine (C_{int} and EC50) for 24 (Fig. 8A and C) and 48 h (Fig. 8B and D). The values are reported for all biological replicates (n = 4). The effect size (difference of medians) and its 95% confidence interval (95% CI) are displayed as a point estimate (black dot) and vertical bar, respectively.

SERS has been often applied to the analysis of intact eukaryotic cells, using metal nanoparticles as nanoprobes to analyze the biochemical composition of cellular environments or to classify tumor cells [36]. This approach requires the internalization of nanoparticles into cells by different techniques such as active uptake, passive diffusion or by mechanical or physical delivery. A possible alternative is to study cells by the SERS analysis of cellular lysates [37]. Using lysates, the spectral information about specific subcellular structures is lost, but the cytosol becomes easily accessible for analysis. On these premises, we propose a direct SERS detection method to monitor the influence of the TPMT activity level on the pharmacokinetics of thiopurine drugs in cell lysates. Thiopurines show intense SERS spectra under most conditions. In fact, thiopurines strongly adsorb onto the Ag NPs surface via the thiol group and the lone pair of electrons on the N atom, with the adsorption sites being affected by the pH of the medium [38]. Mercaptopurine adopts the thiol tautomer form when in solution. At acidic pH values, as in this study, a strong interaction with Ag through the N9 and N3 atoms is suggested for mercaptopurine, with a perpendicular orientation [39]. Also, SERS spectra intensity and Raman shift of thioguanine are affected by pH value [40]. Thioguanine may exist in many tautomeric forms among which, in aqueous solution, the tautomer with the thione and the hydrogen atom in N9 is predominant [40]. Thioguanine may interact with Ag via the sulphur atom and the N1 and N7 atoms may both contribute to this kind of attachment under acidic condition [40].

In SERS spectra obtained for cell lysates treated with mercaptopurine, the most intense characteristic bands of mercaptopurine at 860 cm^{-1}, 1000 cm^{-1}, 1280 cm^{-1} are not visible, indicating that the drug has been completely metabolized. Also, in the SERS signal of cell lysates treated with thioguanine, the most intense characteristic band of thioguanine at 1300 cm^{-1} is not detected. On the other hand, characteristic bands of the TGN metabolites appear in the treated NALM6 cell lysates (but not in the untreated cell lysates) at 439, 534, 876, 913, 1264, 1325, 1506 cm^{-1}, denoting that TGN were formed from the *in vitro* cell metabolism after thiopurine drug incubation. In this study, the determination of a different TPMT enzyme activity is based on the detection of the cytosolic levels of TGMP, TGDP and TGTP that are collectively considered as TGN as they have identical SERS spectra, though they present different intensities of the characteristic bands.

SERS signals of MeMP and MeTGN were not observable, probably because the methyl group at the sulphur atom interfered with the possibility to create a sulphur-metal bond on Ag NPs. Marz A et al. [41] described a lab-on-a-chip SERS method to determine the TPMT activity in lysed red blood cells by analyzing the methylation of mercaptopurine to MeMP, in particular by measuring the difference spectrum of red blood samples spiked with mercaptopurine with or without incubation, representing present or absent TPMT acitivity, to detect the conversion of mercaptopurine to MeMP. However, these authors considered a much higher mercaptopurine concentration than the ones used in this study (1.1 mM *versus* the 0.53–1.44 μM range, respectively). It is therefore likely that in our experimental setting the concentrations of MeMP and MeTGN formed by the *in vitro* cell metabolism are insufficient to be detected by SERS.

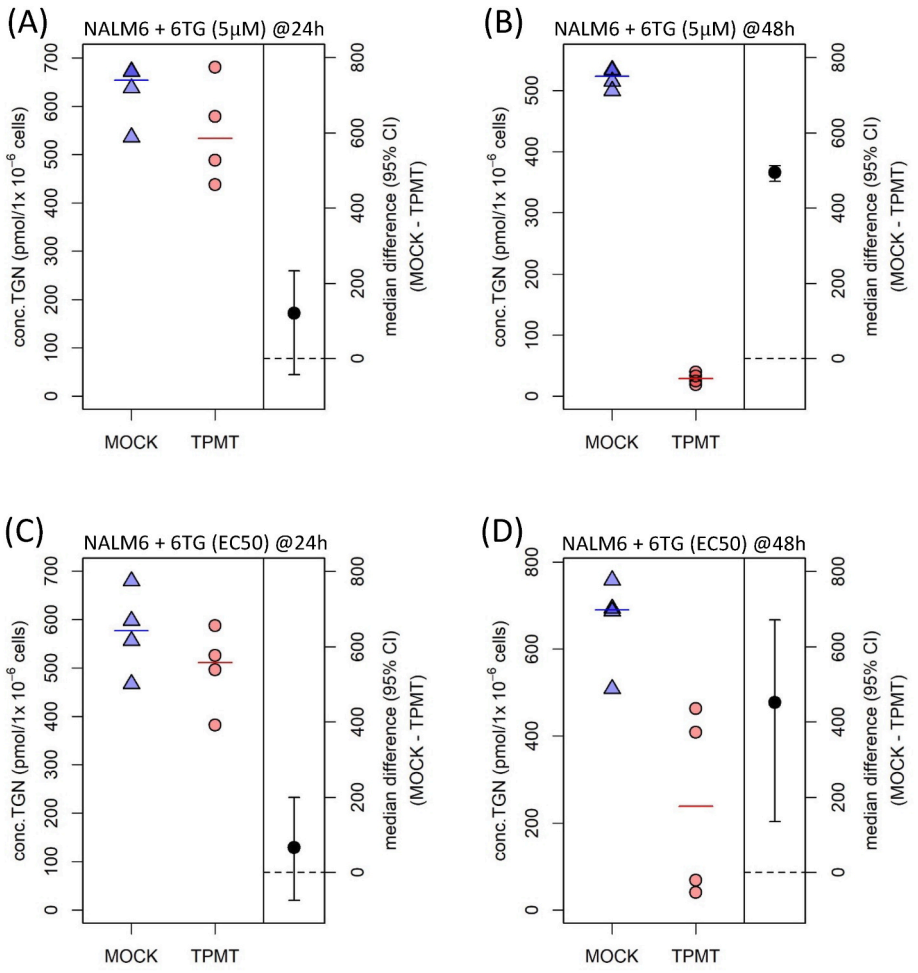

Fig. 9. Gardner-Altman plots of the concentration of all TGN (pmol/1 x 10^6 cells) measured by LC-MS/MS in NALM6 MOCK and TPMT*1 treated with thioguanine (C_{int} and EC50) for 24 (Fig. 9A and C) and 48 h (Fig. 9B and D). The values are reported for all biological replicates (n = 4). The effect size (difference of medians) and its 95% confidence interval (95% CI) are displayed as a point estimate (black dot) and vertical bar, respectively.

A much higher intensity of the characteristic TGN band at 915 cm^{-1} was observed after thioguanine treatment when compared to mercaptopurine exposure. This could be explained by the more direct intracellular activation of thioguanine and the faster formation of TGN compared to the mercaptopurine metabolic pathway that forms many intermediate metabolites, which are TPMT substrates and result in methylated metabolites in detriment of TGN. Also clinical studies results have shown higher TGN levels in patients treated with thioguanine compared to mercaptopurine [42]. This result was then confirmed also by mass-spectrometry analysis.

Considering in particular TGN, that were also analyzed by mass-spectrometry, it emerges that after 24 h of treatment with mercaptopurine (both for EC50 and C_{int}), the most abundant TGN in NALM6 MOCK is TGMP. In NALM6 TPMT*1, TGMP is present in significantly smaller quantities than in NALM6 MOCK. After 48 h of mercaptopurine exposure, there is a decrease in TGN concentrations for both NALM6 MOCK and TPMT*1. As regards the quantification after 24 h of thioguanine treatment, the TGN concentrations are similar between NALM6 TPMT*1 and MOCK but the methylated nucleotide metabolites are already higher in NALM6 TPMT*1. Indeed, after 48 h of thioguanine exposure, the mass-spectrometry assay detects small quantities of TGN in NALM6 TPMT*1, while in NALM6 MOCK it still reveals quite high concentrations of active metabolites, among which the most abundant is TGMP. On the basis of these results, we can conclude that the SERS signal of TGN in treated cell lysates is primarily given by TGMP. Among the methylated thioinosine metabolites, MeTIMP is the most abundant after mercaptopurine treatment at both 24 and 48 h, being much more concentrated in NALM6 TPMT*1 than in NALM6 MOCK at both time points. Methylated thioguanine nucleotides are present in small quantities in NALM6 MOCK both at 24 and 48 h of mercaptopurine treatments. In NALM6 TPMT*1 they are detected in high concentrations at 24 h. At 48 h, there is a difference between 5 μM and EC50, being still high for EC50 treatment, but lower than at 24 h. After thioguanine exposure, MeTGMP is the most abundant methylated metabolite both at 24 and 48 h, much more concentrated in NALM6 TPMT*1 than in NALM6 MOCK.

In conclusion, the study aimed at the development of a direct SERS analysis method to discriminate between cells with different levels of TPMT enzymatic activity upon exposure to mercaptopurine and thioguanine for 24 and 48 h, on the basis of the amount of TGN formed. The influence of TPMT overexpression on the *in vitro* metabolism of thiopurines where highlighted. NALM6 MOCK allows the formation of high concentrations of TGN, detected by SERS with their characteristic peak at 915 cm^{-1}. The overexpression of TPMT, on the contrary, leads to the formation of more methylated metabolites in NALM6 TPMT*1, whose signal is not visible by SERS, to the detriment of TGN. Therefore, NALM6 TPMT*1 have a sharply reduced band at 915 cm^{-1}. Mass-spectrometry analysis confirmed that in NALM6 TPMT *1, mercaptopurine and thioguanine were mainly biotransformed into methylated metabolites, mostly MeTIMP and MeTGN, respectively. The results of mass-spectrometry analysis allowed to determine the different quantities of each TGN; by far, the most abundant is TGMP, and it is undoubtedly the

one that contributes most to the SERS signal.

We reported a direct SERS detection method that could be a tool for the study of thiopurine drug pharmacokinetics in *in vitro* cellular models to qualitatively discriminate between cells that do and do not over-express the TPMT enzyme to evaluate the transfection stability, as an alternative to other more laborious techniques.

However it is important to emphasize that the SERS method described in this study should be regarded as semi-quantitative. Therefore, a direct comparison with the LC-MS/MS results is not feasible. LC-MS/MS provides absolute concentrations of the analytes of interest when the detector is calibrated appropriately. Also for SERS, absolute quantification can theoretically be achieved after proper calibration. The field of absolute quantification using SERS remains, however, an unsolved active area of investigation. Nevertheless, it is worth noting that a strong positive correlation (Spearman's rank correlation coefficient rho = 0.96) exists between absolute quantification of TGMP (pmol/1 x 10^6 cells), obtained by LC-MS/MS, and SERS signal (intensity at 915 cm^{-1}).

This work presents the results of a proof-of-concept study; the subsequent step, which must be taken before the SERS method can be applied to high-throughput, large-scale drug screening test, is a comprehensive investigation of scalability. In future studies, we aim to apply this method to investigate the TPMT activity directly in pediatric patients' leukocytes quantifying TGN formation as an alternative to genetic analysis since genetic variants are not the only cause of altered TPMT activity in patients.

Author Statement

Pagarin Sofia: concenptualization, investigation, formal analysis, writing-original draft; Bolognese Anna: concenptualization, investigation, formal analysis; writing-review & editing; Fornasaro Stefano: data curation, formal analysis, writing-review & editing; Franzin Martina: investigation, writing-review & editing; Hofmann Ute, Schwab Matthias: resources, writing-review & editing; Franca Raffaella, Lucafò Marianna: concenptualization, supervision, writing-review & editing; Decorti Giuliana, Stocco Gabriele: concenptualization, supervision, founding acquisition, writing-review & editing; Bonifacio Alois: supervision, visualization, writing-review & editing.

Declaration of Competing interest

None.

Data availability

Data will be made available on request.

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