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Three Faces of Mercaptopurine Cytotoxicity *in vitro*: Methylation, Nucleotide Homeostasis and Deoxythioguanosine in DNA

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6-thio-2-deoxyguanosine (deoxythioguanosine; dTG); 6-thioguanosine-mono-phosphate (TGMP); 6-thioguanosine-di-phosphate (TGDP); 6-thioguanosine-tri-phosphate (TGTP); 6-methylthioguanosine-mono-phosphate (meTGMP); 6-methylthioguanosine-di-phosphate (meTGDP); 6-methylthioguanosine-tri-phosphate (meTGTP); 6-methylthioinosine-mono-phosphate (meTIMP); 6-methylthioinosine-tri-phosphate (meTITP); 6-thioinosine-mono-phosphate (TIMP); 6-thioinosine-di-phosphate (TIDP); 6-thioinosine-tri-phosphate (TITP).

ABSTRACT

Mercaptopurine (MP) is a cytotoxic thiopurine important for the treatment of cancer and autoimmune diseases. MP and other thiopurine drugs undergo extensive intracellular metabolism but the mechanisms of action are poorly characterised. In particular, it is unknown how different metabolites contribute to cytotoxicity and incorporation of thiopurine bases into DNA. The aim of this study was to ask whether cytotoxicity results from the incorporation of thioguanosine nucleotides into DNA, an alternative thiopurine metabolite, or a combination of factors. Therefore, we measured the cytotoxicity, metabolism, and incorporation of thioguanosine into DNA in response to MP or MP metabolites. Thiopurine metabolites varied in cytotoxicity, with methyl-thioinosine- mono-phosphate and thioguanosine-tri-phosphate the most toxic, and the methyl-thioguanosine nucleotides the least. We show, using liquid chromatography-tandem mass spectrometry, how different metabolites may perturb biochemical pathways, particularly disrupting guanosine nucleotide homeostasis which may contribute to the mechanism of action of thiopurines. Although there was no correlation between metabolite cytotoxicity and the levels of 6-methylthioinosine-mono-phosphate or thioguanosine incorporation into DNA as individual factors, a combined analysis suggested that these factors together had a major influence on cytotoxicity. This study emphasises the importance of enzymes of nucleotide homeostasis, methylation and demethylation in thiopurine effects. These results will facilitate the development of dynamic biochemical models of thiopurine biochemistry which will improve our understanding of mechanisms of action in relevant target tissues.

Introduction

The thiopurine drugs, azathioprine, mercaptopurine (MP) and 6-thioguanine have been used as immunosuppressants since the early 1950s for treatment of inflammatory bowel disease (IBD), childhood acute lymphoblastic leukaemia, autoimmune hepatitis, rheumatic disease and as a drug to reduce organ transplant rejection (Coulthard and Hogarth, 2005; Fotoohi et al., 2010). Thiopurines are pro-drugs and have to be extensively metabolised to exert their cytotoxic effect. Azathioprine is non-enzymatically reduced to MP and an imidazole group. Both MP and 6-thioguanine undergo metabolism before exerting cytotoxicity: both drugs generate thioguanosine nucleotides via hypoxanthine guanine phosphoribosyltransferase (HPRT), but the metabolism of MP is more complex with a different range of metabolites compared to 6-thioguanine. Deoxythioguanosine-tri-phosphate, a metabolite produced by MP or 6-thioguanine, is a substrate for DNA polymerases and estimates of steady-state levels of deoxythioguanosine (dTG) in DNA of patients suggest that during replication between 0.01 and 0.1% of DNA guanines (around 10^5 to 10^6 per cell) may be substituted by the thiobase (Warren et al., 1995). In addition, MP or its metabolites can be diverted from pathways of nucleotide incorporation by xanthine oxidase-mediated inactivation to thiouric acid, or by thiopurine methyltransferase (TPMT)-mediated methylation of thiopurine nucleotides or thioinosine nucleotide precursors (Coulthard and Hogarth, 2005; Fotoohi et al., 2010; Coulthard, 2012) (Fig. 1).

Several mechanisms of action have been elucidated to explain the therapeutic mechanisms of thiopurines as immunosuppressants or anti-leukaemic drugs, and these include the inhibition of *de novo* purine synthesis (DNPS) (Dervieux et al., 2001; Coulthard et al., 2002), alterations in DNA methylation state via decreased levels of DNA (cytosine-5)-methyltransferase 1 (DNMT1) (Coulthard et al., 2011), the disruption of GTP signalling, and incorporation of thioguanosine nucleotides as fraudulent bases into the DNA (Tiede et al., 2003). The latter causes DNA-protein cross-links, single strand breaks, inter-strand cross-links and sister chromatid exchanges (Maybaum

and Mandel, 1981; Maybaum and Mandel, 1983; Pan and Nelson, 1990) resulting in activation of the mismatch repair system (Swann et al., 1996) and induction of apoptosis. Furthermore, the inhibition of Rac1 activation by the binding of TGTP instead of GTP to Rac1, induces apoptosis via a mitochondrial pathway (Tiede et al., 2003).

Although therapeutic responses in leukaemia correlate with levels of dTG incorporation in target cells (Ebbesen et al., 2013), the relationships between clinical outcome and other potential mechanisms of response such as Rac1 inhibition or DPNS are unknown, particularly for MP (or its precursor drug azathioprine) which has greater metabolic complexity than 6-thioguanine. Furthermore, in the context of immunosuppression, the amount of dTG in DNA does not appear to be a biological marker of clinical response in IBD (Coulthard et al., 2017). Key issues for understanding the therapeutic mechanisms of MP are how different metabolites and their rates of production contribute to cytotoxicity and the incorporation of dTG into DNA, and whether these are separable consequences of different pathways of MP metabolism. These issues need to be addressed in an isogenic background to avoid cell-specific variation in expression of enzymes or other cellular characteristics which would otherwise confound interpretation. MP is used to treat childhood ALL as part of maintenance therapy; therefore, we have employed MOLT-4 cells, a T-cell acute lymphoblastic leukaemia cell line, as an experimental model. For this proof-of-principle study we developed a new liquid chromatography-mass spectrometry (LS-MS/MS) method to investigate the metabolism of MP and its metabolites, and to test the link between cytotoxicity, thiopurine metabolites and incorporation of fraudulent bases into DNA.

Materials and Methods

Cell culture

MOLT-4 (T-cell acute lymphoblastic leukaemia cells, derived from authenticated stocks in this institute, were maintained in RPMI 1640 (Sigma-Aldrich, Poole, UK) containing 10% foetal calf

serum (Sigma-Aldrich) at 37 °C, 5% CO_2, and were tested for mycoplasma infection every 2 months. For viability assays, repeated on three separate occasions, cells were seeded into 96-well plates at 2 x 10^3/100 µL and after 24 h, serially-diluted thiopurine metabolites (a single metabolite per plate) were added to the cells; cell viability was determined after 72 h using the MTS assay (CellTiter 96® AQueous Assay, Promega™, Southampton, UK) according to the manufacturer's protocol.

Twelve thiopurine metabolites were commercially available (Jena Bioscience, Jena, Germany): 6-thioguanosine-mono-phosphate (TGMP), 6-thioguanosine-di-phosphate (TGDP), 6-thioguanosine-tri-phosphate (TGTP), 6-methylthioguanosine-mono-phosphate (meTGMP), 6-methylthioguanosine-di-phosphate (meTGDP), 6-methylthioguanosine-tri-phosphate (meTGTP), 6-methylthioinosine-mono-phosphate (meTIMP), 6-methylthioinosine-di-phosphate (meTIDP), 6-methylthioinosine-tri-phosphate (meTITP), 6-thioinosine-mono-phosphate (TIMP), 6-thioinosine-di-phosphate (TIDP) and 6-thioinosine-tri-phosphate (TITP). IUPAC names are given in Supplemental Information. For drug sensitivity assays, cells were exposed to each thiopurine metabolite individually on three separate occasions for 72 h (three-doubling times); this is the time required for incorporation of thioguanosine nucleotides into the DNA of replicating cells and the minimum exposure time to elicit cell death by thiopurine drugs at the EC_{50} concentrations determined by the MTS assay. The concentrations of MP used were similar to plasma concentrations (1.3 µM, range 0.75 - 2.2 µM) in children with ALL treated with standard doses of MP (100 mg/m^2) (Estlin, 2001).

Cell pellets for LC-MS/MS assays were generated by seeding MOLT-4 cells at a density of 1.5 x 10^5/mL (total of 1.5 x 10^6) and thiopurine metabolites added to the appropriate concentrations. After 72 h, cells were pelleted by centrifugation, resuspended and washed twice in PBS to remove free external drug or metabolites, and washed cell pellets stored at -80 °C prior to extraction for analysis by LC-MS/MS. Pellets were generated for all metabolite-treated and control (no metabolite) cells in three independent experiments.

Sample preparation for LC-MS/MS assay of deoxythioguanosine in DNA (dTG^{DNA})

Cell pellets were re-suspended in 350 µL of Buffer B (400 mM Tris, 60 mM EDTA, 150 mM NaCl) and 90 µL of 5 M sodium perchlorate. Samples were mixed at room temperature for 10 min, or until the pellet was completely dissolved after which they were incubated at 65 °C for 45 min. An equal volume of chloroform was added to each sample and mixed for 20 min at room temperature, followed by centrifugation at 432 g for 10 min at 4 °C. The top layer was then removed and DNA precipitated by adding 2.5 volumes of ethanol and samples centrifuged at 16,000 g. Precipitated DNA was re-suspended in 5 mM of Tris-HCl, pH 8, by incubation overnight at 50 °C.

Analysis of dTG^{DNA} was done using a previously-published method (Coulthard et al., 2016). In outline, DNA was diluted to 0.2 µg/mL in a total volume of 100 µL of 1 µM $MeMPd_3$ and denatured by heating to 100℃ for 5 min. After being chilled on ice for 2 min, 10 µL of 10x digestion buffer (500 mM sodium acetate, 10 mM $MgCl_2$ pH5.3) and 5 µL of 0.12 U/µL nuclease P1 was added and incubated for 1 h at 50 ℃. Finally, 20 µL Tris-HCl and 1 µL of alkaline phosphatase (1 U/mL) were added to each sample, incubated for 30 min at 40 °C and samples then diluted either 1/10 or 1/100 for analysis by LC-MS/MS.

Sample preparation for LC-MS/MS assay of cytosolic thiopurine metabolites

MOLT-4 cell pellets were defrosted on ice and ice cold propanol (200 µL) immediately added. Propanol was evaporated from the cells using a TurboVap LV automated evaporation system (Biotage, Hengoed, UK) with nitrogen gas (5 psi). Once completely dry, 200 µL of deionised water was added, samples vortexed and centrifuged 16,000 g for 10 min. The supernatant was transferred into a HPLC glass insert for subsequent LC-MS/MS analysis.

LC-MS/MS and reagents

The thiopurine metabolite standards, as listed above, were from Jena Bioscience. 6-Thio-2-deoxyguanosine standard (deoxythioguanosine; dTG) was from Carbosynth (Compton, UK). Deuterated methyl mercaptopurine was from Toronto Research Chemicals Inc. (Toronto, Canada). HPLC grade acetic acid was from Fisher Scientific (Loughborough, UK). Lymphoprep™ was from Axis-Shield and Pierce BCA kit was from Thermo Scientific (Cramlington, UK). All other reagents were from Sigma-Aldrich. Chromatographic separations were performed with a Shimadzu Prominence UFLC (Shimadzu, Kyoto, Japan).

An API4000 triple quadrupole MS/MS (Applied Biosystems, California, USA) was used for analysis with electrospray ionisation (ESI) performed in positive ion mode using nitrogen gas. Optimisation of MS/MS parameters for all analytes was performed by selecting precursor ions and determining the most prominent four product ions. The three best were then further optimised for fragmentation and voltage parameters until the most abundant and robust product ion could be ascertained. Quantification of analytes was performed in multiple reaction monitoring (MRM) mode: mass transitions and optimised MS/MS parameters are given in Supplemental Table 1 and validation methods in Supplemental Information and Supplemental Table 2. Analyst® software v1.5 (AB SCIEX, Framingham, USA) was used for sample analysis, peak integration and analyte quantification.

LC-MS/MS analysis of cytosolic thiopurine metabolites

Chromatographic separation of thiopurine metabolites was achieved using a Clarity Oligo-WAX column (150 mm x 4.6 mm) and SecurityGuard Oligo-WAX guard column (4 x 3 mm) both from Phenomenex (Cheshire, UK) maintained at 30 °C. Analytes were eluted with mobile phases of 0.01 M aqueous ammonium acetate pH 8.0 (A) and 0.01 M aqueous ammonium acetate pH 10.01 (B). The mobile phase system consisted of a starting condition at 90% A followed by a 0.5 min gradient from 90% A to 80% A at 1.5 min. A 2 min gradient from 80% A to 0% A was carried out at 3.5 min with those conditions maintained until 13.5 min when the column was returned to 10% A in a 0.5

min gradient. The flow rate was 0.7 mL/min and a post column flow splitter was utilised to divert 70% of mobile phase to waste and improve ionisation. Standards and samples were injected in a volume of 50 µL. Standards were freshly diluted from a secondary dilution of stock on the day of analysis, and the secondary dilutions were used within six months of preparation from the primary stocks received from the manufacturer; the standards were stable under this protocol. Optimum settings for MS/MS detection were: curtain gas, 10; ion source gas 1, 40; ion source gas 2, 50; ion spray voltage, 5500; collision gas, 6; entrance potential, 10; ionisation temperature, 400 °C. Examples of LC-MS/MS profiles for standards and samples are shown in Fig.2.

LC-MS/MS analysis of deoxythioguanosine in DNA (dTG^{DNA})

Chromatographic separation of dTG and deoxyadenosine from P1 nuclease digestion of DNA was achieved using a XSelect HSS T3 3.5 µm 4.6x100 mm (Waters) XSelect HSS T3, a VanGuard Cart 3.5 µm 3.9 x 5 mm guard column maintained at 30 ℃, and mobile phases of aqueous 0.05% formic acid and 0.05% formic acid in acetonitrile (Coulthard et al., 2016). Standards were prepared from stock solutions of 100 µg/mL deoxyadenosine and 2000 ng/mL dTG; standards and samples were injected in a volume of 50 µL (Coulthard et al., 2016). Two standard curves and three sets of controls for each metabolite were included in each assay.

***In silico* modelling**

Interconversion between thioguanosine nucleoside mono-, di- and tri-phosphates was modelled using the biochemical simulation software CoPaSi version 4.12 (Hoops et al., 2006). Rate constants, based on values in the BRENDA enzyme database (www.brenda-enzymes.org) (Placzek et al., 2017), were fixed and V for rate equations in the model fitted to experimental data.

Data analysis

Viability data were obtained in three separate experiments for each compound. Data were blank (medium but no cells) corrected and expressed as a proportion of control cells treated with vehicle only. Mixed-effects models to account for random differences between experiments were used to estimate the mean and standard error (SE) for the upper asymptote, slope and EC_{50} from 3-parameter logistic curves with the 'nlme' package in R version 3.1.2 (R Core Team, 2016). EC_{50} was defined with respect to the maximum and minimum of the logistic curves rather than the viability of untreated control cells; some of the compounds showed hormetic effects by increasing cell growth (viability) at low doses and this was reflected by fitted upper asymptotes > 100% of control. To avoid over-fitting, the lower asymptote was fixed at zero for data where viability was approaching zero but the lower asymptote was just outside the data range, but otherwise was fixed on estimates obtained from a 4-parameter curve fit with all data using package 'drc' in R (Ritz et al., 2015). For two drugs where mixed-effects fits did not converge to a solution, 4-parameter logistic curves were fitted to each experiment using drc and the mean of separate EC_{50} values calculated. Data are expressed as means ± SEM. Partial correlation and principal components analyses were carried out using the R packages 'ppcor' and 'FactoMineR', respectively.

Results

Cytotoxicity of thiopurine metabolites

To assess the cytotoxicity of individual thiopurine metabolites, an extended incubation time of 72 h, representing approximately three cell doublings, was used to assess the effects on cell viability. This timescale was also used for assessing the role of dTG incorporation into DNA as a mechanism of cytotoxicity of the parent thiopurine drug or its metabolites. After incubation with cells for 72 h, the parent drug, MP, had an EC_{50} of the order of 3 μM (Fig. 3; Table 1). With the exception of the thioinosine di- and tri-phosphates and the methylated thioguanosine nucleotides, MP metabolites were more toxic, with EC_{50} values ranging from 0.6 to < 0.2 μM. The thioguanosine nucleotides

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varied in cytotoxicity with TGTP the most toxic (EC_{50} 0.265 μM) and TGMP the least (EC_{50} 0.619 μM). The methylated thioguanosine nucleotides had the lowest toxicities of all, in spite of being able to enter cells (see below), and only meTGTP elicited a cellular response, albeit at a very high concentration (EC_{50} 15.37 μM). TIMP is a direct derivative of the parent drug, 6-MP, via HPRT and had a relatively high cytotoxicity comparable to TGTP, but this was increased by methylation as meTIMP had the highest cytotoxicity of all the metabolites tested (EC_{50} 0.185 μM; Table 1). Methylation also doubled the cytotoxicity of TITP, but only had a small effect on cytotoxicity of TIDP (Table 1).

Metabolism of mercaptopurine metabolites

MP and thioguanosine nucleoside phosphate metabolites. Cells were incubated with MP or individual metabolites at their EC_{50} concentrations for 72 h before LC-MS/MS analysis of intracellular metabolite levels. TIMP, TIDP and TITP were not measured as there was no separation by LC and the major ions detected by MS/MS were the same for each compound. After incubation with MP, meTIMP was the major cellular metabolite, together with residual amounts of parent drug. Thioguanosine nucleoside phosphates were present at lower levels, with TGMP the most abundant and lower levels of TGDP and TGTP (Fig. 4). For cells incubated with thioguanosine nucleoside phosphates, treatment with either TGMP or TGDP produced similar distributions of all three thioguanosine phosphates, with TGDP predominating in both cases; the methylated equivalents had similar profiles but at levels one-fifth or lower of the unmethylated thioguanosine nucleotides (Fig. 4). Conversely, in cells incubated with TGTP, the profile of thioguanosine nucleotides differed markedly, with TGMP in greatest abundance and substantially lower levels of TGDP and the parent TGTP. Cells treated with MP had a distribution of thioguanosine nucleoside phosphates substantially biased towards TGMP similar to TGTP-treated cells, (Fig. 4A,B).

To provide insight into potential mechanisms underlying differences in thioguanosine nucleotide profiles between cells treated with TGTP, and either TGMP or TGDP, steady-state

thioguanosine nucleotide levels were modelled as a simple network of the three thioguanosine nucleotides in a closed system with enzyme activity representing guanylate kinase, nucleotide diphosphate kinase, GTPase, ATP apyrase (acting on TGTP), and guanosine diphosphatase (Fig. 4C). At steady-state, an initial model was a good fit to experimental data for cells treated with TGMP or TGDP with respect to the proportions of thioguanosine nucleotides, but not for the experimental data for cells treated with TGTP. Refitting the model separately to experimental data for TGTP-treated cells showed that a 16-fold increase in guanosine diphosphatase activity would be sufficient to account for the thioguanosine nucleotide profile observed for cells treated with TGTP (Fig. 4D).

Methyl-thioguanosine nucleoside phosphate metabolites. Treatment of cells with meTGMP resulted in some demethylation to TGMP after 3 days, and some possible conversion to lower levels of meTGDP and meTGTP, giving a distribution similar to the unmethylated thioguanosine nucleotides in Fig. 4A,B. Conversely, meTGDP was converted mainly to meTGMP. For cells treated with meTGTP, there was substantial dephosphorylation to meTGMP and demethylation to TGMP with TGMP the main metabolite remaining (Fig. 5A), and a similar distribution of methylated thioguanosine nucleoside phosphates to the unmethylated equivalents (Fig. 4A,B). Thus, both dephosphorylation and demethylation reactions are involved in the metabolism of the methylated thioguanosine nucleoside phosphates.

Thioinosine and methyl thioinosine nucleoside phosphate metabolites. After incubation with TIMP, there was substantial methylation to meTIMP; TIMP was also metabolised to TGMP and low levels of methylated thioguanosine nucleotides were also produced (Fig. 5B), as would be predicted from known biochemical pathways. For TIDP and TITP, in contrast to TIMP, there was very little conversion to meTIMP and only very small amounts of TGMP were detectable (Fig. 5B).

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MeTIMP was the major metabolite after incubation with meTIMP, meTITP or meTIDP with no other metabolites detectable other than low levels of residual treatment metabolite (Fig. 5C). The presence of low levels of meTIDP and meTITP after treatment with meTIMP may represent low-level contaminants of ≤ 3% in the commercial source of meTIMP used; these compounds were not detectable above background at the concentrations of meTIMP analytical standards used. The results show that meTIDP and meTITP were efficiently dephosphorylated to meTIMP but without evidence for demethylation. Although meTIMP was the most toxic metabolite in viability assays, there was no correlation between metabolite EC_{50} in viability assays (Table 1) and the levels of meTIMP produced from each metabolite in treated cells (Spearman rank correlation, P =0.2873).

Incorporation of thioguanosine nucleotides into DNA

In separate experiments, MOLT4 cells were treated with parent drug or each of the metabolites (except TIDP and TITP; Fig. 6) at EC_{50} concentrations for 72 h and DNA extracted for assay of dTG. DNA-incorporated dTG was detectable significantly above background (ANOVA, Dunnett's post-hoc test, $P < 0.05$; Fig. 6) only in cells treated with MP ($P < 0.001$), TGDP ($P < 0.001$), TGTP ($P = 0.03$) or TIMP ($P = 0.006$). There was no significant correlation between dTG^{DNA} and metabolite EC_{50} in viability assays (Spearman rank correlation, P =0.3614). Apart from the low dTG^{DNA} after TGMP treatment, these results confirm the expected route of incorporation of thioguanosine metabolites of MP into DNA via TIMP, TGDP and TGTP, and show that meTIMP cytotoxicity was independent of dTG^{DNA}.

Data integration

The data for cytotoxicity (EC_{50}) to different metabolites, dTG^{DNA} and metabolite levels after treatment of cells with different metabolites for 72 h, were summarised using partial correlation and principal components analyses. To reduce the number of variables, measurements of mono-, di- and tri-phosphates were combined to give thioguanosine nucleoside phosphates (TGXP), methyl-

thioguanosine nucleoside phosphates (meTGXP) and methyl-thioinosine nucleoside phosphates (meTIXP). There was a significant partial correlation between TGXP and meTGXP (P= 0.005), as might be predicted; the partial correlation between dTG^{DNA} and TGXP approached significance (P=0.065) and such a relationship would also be expected. However, there were no significant partial correlations between EC_{50} and either dTG^{DNA} or thioguanosine metabolites (P > 0.1). In a principal component analysis of dTG^{DNA} and thioguanosine metabolites as independent variables, 74% and 18% of the variance was accounted for by two principal components (PC1 and PC2, respectively; Supplemental Table 3). PC1 had major contributions from dTG^{DNA}, TGXP and meTGXP, whereas meTIXP was the dominant component (82.7%) of PC2 (Table 2), with a small contribution from dTG^{DNA} (13.6%). There was no significant correlation of EC_{50} with PC1 (P > 0.5; Pearson correlation coefficient = -0.24; t_8 = -0.66), but the correlation between EC_{50} and PC2 approached significance (P = 0.059; Pearson correlation coefficient = -0.62; t_8= -2.21), suggesting that methyl-thioinosine nucleoside phosphates might be key drivers of cell toxicity.

Discussion

Although in the past many studies on the biochemistry of thiopurines have focussed on red blood cells, such studies will not capture the complexity of biological responses to MP in nucleated cells, the biological targets for thiopurine therapy (Duley and Florin, 2005). Reliable measurement of thiopurine metabolites in isolated blast cells from leukaemic patients is notoriously difficult as exemplified by the paucity of published methods. A relevant *in vitro* model is important for the development of appropriate analytical methods for elucidating the contribution of different thiopurine metabolites to cytotoxicity and the mechanism of action of MP. In this respect, the present study is unique in assessing how thiopurine cytotoxicity and incorporation of fraudulent bases into DNA is related to the levels of different thiopurine metabolites.

The results emphasise that the cytotoxicity of thiopurines on nucleated cells will be combinatorial effects of different mechanisms. The incorporation of thioguanosine nucleotides into DNA promotes cell-cycle arrest (Karran, 2006) and increases the chance of DNA strand breaks (Fairchild et al., 1986; Lennard, 1992). These effects will account for some of the cytotoxicity of MP and metabolites such as TGDP. Overall, the incorporation of dTG into DNA may be a minor mechanism of cytotoxicity in MOLT4 cells. Recent studies have failed to find a good correlation between dTG incorporation into DNA and clinical efficacy as immunosuppressants (Coulthard et al., 2016; Coulthard et al., 2017), but the converse has been established for acute lymphoblastic leukaemia (Nielsen et al., 2017). However, it is important to recognise the very different contexts of disease biology between uses of thiopurines as anti-cancer drugs versus thiopurines as immune system modulators.

MP can exert a cytotoxic effect in cells with high levels of TPMT without measurable levels of dTG^{DNA} (Coulthard et al., 2002) and our analyses of thiopurine metabolites after treating cells with the parent drug or its known metabolites highlight perturbations to biochemical pathways which may underlie alternative mechanisms of cytotoxicity, even for leukaemia cells *in vitro*. LC-MS/MS data show that all metabolites were able to enter the cells, and for simplicity in interpretation we assume comparable rate constants.

MeTIMP was the most toxic metabolite in viability assays and was the major metabolite in MP-treated cells after 72 h. There was no evidence for metabolism of meTIMP to thioguanosine nucleotides for incorporation into DNA, or evidence of demethylation to other thio-nucleotide metabolites. From other studies there is direct evidence that meTIMP is an inhibitor of phosphoribosyl pyrophosphate amidotransferase (Tay et al., 1969; Vogt et al., 1993) and will reduce cell viability as a consequence of the inhibition of DNPS (Coulthard et al., 2002; Coulthard et al., 2011) and decreased RNA synthesis. Inhibition of DNPS will increase incorporation of dTG into DNA as a consequence of a reduction in the endogenous nucleotide pool (Bokkerink et al., 1993; Ebbesen et al., 2013); therefore, inhibition of DNPS via meTIMP in MOLT4 cells will be a

substantial factor in the cytotoxic effects of MP, necessitating reliance on the nucleotide salvage pathway to sustain DNA replication (Coulthard et al., 2011). MOLT4 cells express the enzyme methylthioadenosine phosphorylase (Yu et al., 1997), an enzyme required for the salvage of adenine and methionine, but cells in which the MTAP gene is deleted are more sensitive to MeTIMP because they lack the ability to ameliorate the effects of DNPS inhibition via nucleotide salvage (Coulthard et al., 2011). The purine salvage pathway has relatively low activity in lymphoblasts (Bokkerink et al., 1993) and meTIMP as a major MP metabolite is likely to play a clinical role in reducing levels of endogenous nucleotides, therefore enhancing the incorporation of dTG into DNA (Hedeland et al., 2010; Ebbesen et al., 2013). MeTIMP also contributes to a decrease in DNA methyltransferase levels and global demethylation (Hogarth et al., 2008).

The amounts of meTIDP and meTITP detected as MP metabolites were very low compared to meTIMP, and were also low in cells treated with these methylthioinosine nucleotides, indicating effective dephosphorylation to meTIMP and suggesting that the *in vitro* cytotoxicity of these compounds was due to conversion to meTIMP. Although it has been stated that TITP is a substrate for TPMT (Stocco et al., 2009; Seinen et al., 2013) the evidence on which this is based is not clear and in our study there was no indication of substantial methylation of TIDP or TITP. Bearing in mind that TIXPs were not measured, there was no evidence that meTIMP was demethylated to TIMP leading to thioguanosine nucleoside phosphate synthesis. With respect to the thioinosine nucleoside phosphates, TIMP was the most cytotoxic presumably as a result of methylation to meTIMP but with a contribution from dTG^{DNA} via metabolism to TGMP.

In addition to meTIMP, MP was metabolised to the three thioguanosine nucleoside phosphates, with TGMP in excess over TGDP and TGTP, a pattern also produced by treating cells with TGTP but not TGDP or TGMP. A higher level of guanosine diphosphatase activity in response to TGTP or MP compared to TGDP or TGMP could account for the comparative effects of the different thioguanosine nucleoside phosphates in these experiments. However, the ratios of thioguanosine nucleotides in treated cells were markedly different to an expectation based on the

endogenous distribution of guanosine nucleotides in normal cells where guanosine-tri-phosphate would normally be in a ~10-fold excess over the mono- and di-phosphates (Traut, 1994). With reference to a simple dynamic model of the three thioguanosine nucleoside phosphates (Fig. 4), this implies a substantial reduction in guanylate kinase and nucleoside diphosphate kinase activities in MOLT-4 cells as a result of MP or thioguanosine nucleotide treatment, accompanied in MP- or TGTP-treated cells by a possible induction of guanosine diphosphatase activity. Thus, changes in endogenous guanosine nucleotide homeostasis, potentially with substantial reductions in endogenous GTP levels as a result of thioguanosine nucleotide-mediated changes in enzyme activities, could be responsible for the inhibition of Rac1 signalling leading to apoptosis in mercaptopurine-treated cells (Tiede et al., 2003). This is an area of substantial importance for future investigation.

The thioguanosine nucleoside phosphates were all present as methylated and un-methylated metabolites. MeTGMP is 12-fold less potent than meTIMP as a DNPS inhibitor (Allan and Bennett, 1971), but all three methylated thioguanosine nucleotides had substantially less cytotoxicity that their unmethylated equivalents. Therefore, methylation of thioguanosine nucleoside phosphates via TPMT is an effective mechanism for reducing the toxicities of these metabolites. In contrast to the lack of evidence for demethylation of methylthioinosine nucleotides, there was clear evidence for demethylation of meTGMP. Most of the meTGDP and meTGTP ended up as either meTGMP or TGMP, consistent with effective demethylation and guanosine diphosphatase activity. There are few studies on the demethylation of meTGMP other than in the context of DNA, but in view of the *in vitro* evidence, this needs to be considered as a biologically-relevant activity.

All the thioguanosine nucleoside phosphates resulted in dTG^{DNA} in treated cells, although this was not significantly above background with TGMP, and was greatest with TGDP, as would be expected from the role of GDP as a precursor for dGTP synthesis for DNA replication (Traut, 1994). However, other mechanisms of cytotoxicity are clearly involved as TGTP was the most cytotoxic despite the lower dTG incorporation into DNA compared to TGDP. This greater

cytotoxicity could result from greater disruption to Rac1 signalling implied by the lower levels of TGTP in TGTP-treated cells.

The lack of correlation between the cytotoxicity of different thiopurine metabolites and the meTIMP or dTG^{DNA} produced by cellular metabolism implies that at least three mechanisms may contribute to the biological activity of MP: meTIMP cytotoxicity by inhibition of DNPS or other cellular functions, reduction in viability as a result of dTG incorporation into DNA, and inhibition of GTP signalling by changes in enzymes important for GTP homeostasis. This is supported by the principal components analysis, which highlights the strong contribution of methylthioinosine nucleoside phosphates to cytotoxicity in combination with dTG^{DNA} in MOLT4 cells. With respect to the parent drug, cell-, tissue- and individual-specific variation in TPMT expression and activity, dependence on DNPS, the status of GTP homeostasis, DNA repair capacity and the rates of drug and metabolite efflux (Wielinga et al., 2002) will all affect the contributions of different mechanisms to biological effects. These mechanisms are amenable, with the availability of sufficient biochemical information, to modelling individually with integration into cell behaviour models, an approach which will facilitate improvements in the clinical use and efficacy of thiopurine drugs. Such an approach would benefit by a deeper understanding of the biochemical pathways of thiopurine metabolism. This study emphasises the importance of enzymes of nucleotide homeostasis, methylation and demethylation in those pathways and will facilitate the development of dynamic biochemical models of thiopurine biochemistry.

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Authorship Contributions

Participated in research design: Coulthard, Redfern.

Conducted experiments: Coulthard, McGarrity, Sahota, Berry.

Contributed new reagents or analytic tools: Berry, Coulthard, McGarrity.

Performed data analysis: Coulthard, McGarrity, Sahota, Berry, Redfern.

Wrote or contributed to the writing of the manuscript: Coulthard, McGarrity, Sahota, Berry, Redfern.

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References

Allan PW and Bennett LL, Jr. (1971) 6-Methylthioguanylic acid, a metabolite of 6-thioguanine. *Biochem Pharmacol* **20:**847-852.

Bokkerink JP, Stet EH, De Abreu RA, Damen FJ, Hulscher TW, Bakker MA, and van Baal JA (1993) 6-Mercaptopurine: cytotoxicity and biochemical pharmacology in human malignant T-lymphoblasts. *Biochem Pharmacol* **45:**1455-1463.

Coulthard S (2012) Mechanism of Thiopurine Action. *Assoc Med Bras* **58:**18-23.

Coulthard S and Hogarth L (2005) The thiopurines: an update. *Investigational New Drugs* **23:**523-532.

Coulthard SA, Berry P, McGarrity S, Ansari A, and Redfern CPF (2016) Liquid chromatography-mass spectrometry for measuring deoxythioguanosine in DNA from thiopurine-treated patients. *J Chromatogr B Analyt Technol Biomed Life Sci* **1028:**175-180.

Coulthard SA, Berry P, McGarrity S, McLaughlin S, Ansari A, and Redfern CPF (2017) Azathioprine with Allopurinol: Lower Deoxythioguanosine in DNA and Transcriptome Changes Indicate Mechanistic Differences to Azathioprine Alone. *Inflam Bowel Diseases*.

Coulthard SA, Hogarth LA, Little M, Matheson EC, Redfern CP, Minto L, and Hall AG (2002) The effect of thiopurine methyltransferase expression on sensitivity to thiopurine drugs. *Mol Pharmacol* **62:**102-109.

Coulthard SA, Redfern CP, Vikingsson S, Lindqvist Appell M, Skoglund K, Jakobsen Falk I, Hall AG, Taylor GA, and Hogarth LA (2011) Increased sensitivity to thiopurines in methylthioadenosine phosphorylase-deleted cancers. *Mol Cancer Ther*.

Dervieux T, Blanco JG, Krynetski EY, Vanin EF, Roussel MF, and Relling MV (2001) Differing contribution of thiopurine methyltransferase to mercaptopurine versus thioguanine effects in human leukemic cells. *Cancer Res* **61:**5810-5816.

Duley JA and Florin THJ (2005) Thiopurine therapies - Problems, complexities, and progress with monitoring thioguanine nucleotides. *Ther Drug Monit* **27:**647-654.

Ebbesen MS, Nersting J, Jacobsen JH, Frandsen TL, Vettenranta K, Abramsson J, Wesenberg F, and Schmiegelow K (2013) Incorporation of 6-thioguanine nucleotides into DNA during maintenance therapy of childhood acute lymphoblastic leukemia-the influence of thiopurine methyltransferase genotypes. *J Clinical Pharmacol* **53:**670-674.

Estlin EJ (2001) Continuing therapy for childhood acute lymphoblastic leukaemia: clinical and cellular pharmacology of methotrexate, 6-mercaptopurine and 6-thioguanine. *Cancer Treat Rev* **27:**351-363.

Fairchild CR, Maybaum J, and Kennedy KA (1986) Concurrent unilateral chromatid damage and DNA strand breakage in response to 6-thioguanine treatment. *Biochem Pharmacol* **35:**3533-3541.

Fotoohi AK, Coulthard SA, and Albertioni F (2010) Thiopurines: factors influencing toxicity and response. *Biochem Pharmacol* **79:**1211-1220.

Hedeland RL, Hvidt K, Nersting J, Rosthoj S, Dalhoff K, Lausen B, and Schmiegelow K (2010) DNA incorporation of 6-thioguanine nucleotides during maintenance therapy of childhood acute lymphoblastic leukaemia and non-Hodgkin lymphoma. *Cancer Chemother Pharmacol* **66:**485-491.

Hogarth LA, Redfern CPF, Teodoridis JM, Hall AG, Anderson H, Case MC, and Coulthard SA (2008) The effect of thiopurine drugs on DNA methylation in relation to TPMT expression. *Biochem Pharmacol* **76:**1024-1035.

Hoops S, Sahle S, Gauges R, Lee C, Pahle J, Simus N, Singhal M, Xu L, Mendes P, and Kummer U (2006) COPASI--a COmplex PAthway SImulator. *Bioinformatics* **22:**3067-3074.

Karran P (2006) Thiopurines, DNA damage, DNA repair and therapy-related cancer. *Brit Med Bull* **79-80:**153-170.

Lennard L (1992) The clinical pharmacology of 6-mercaptopurine. *Eur J Clinical Pharmacol* **43:**329-339.

Maybaum J and Mandel HG (1981) Differential chromatid damage induced by 6-thioguanine in CHO cells. *Exp Cell Res* **135:**465-468.

Maybaum J and Mandel HG (1983) Unilateral chromatid damage: a new basis for 6-thioguanine cytotoxicity. *Cancer Res* **43:**3852-3856.

Nielsen SN, Grell K, Nersting J, Abrahamsson J, Lund B, Kanerva J, Jonsson OG, Vaitkeviciene G, Pruunsild K, Hjalgrim LL, and Schmiegelow K (2017) DNA-thioguanine nucleotide concentration and relapse-free survival during maintenance therapy of childhood acute lymphoblastic leukaemia (NOPHO ALL2008): a prospective substudy of a phase 3 trial. *Lancet Oncol* **18:**515-524.

Pan BF and Nelson JA (1990) Characterization of the DNA damage in 6-thioguanine-treated cells. *Biochem Pharmacol* **40:**1063-1069.

Placzek S, Schomburg I, Chang A, Jeske L, Ulbrich M, Tillack J, and Schomburg D (2017) BRENDA in 2017: new perspectives and new tools in BRENDA. *Nucleic Acids Res* **45:**D380-D388.

R Core Team (2016) R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria.

Ritz C, Baty F, Streibig JC, and Gerhard D (2015) Dose-Response Analysis Using R. *PLoS One* **10**.

Seinen ML, van Asseldonk DP, de Boer NKH, Losekoot N, Smid K, Mulder CJJ, Bouma G, Peters GJ, and van Bodegraven AA (2013) The effect of allopurinol and low-dose thiopurine combination therapy on the activity of three pivotal thiopurine metabolizing enzymes: Results from a prospective pharmacological study. *J Crohns & Colitis* **7:**812-819.

Stocco G, Cheok MH, Crews KR, Dervieux T, French D, Pei D, Yang W, Cheng C, Pui CH, Relling MV, and Evans WE (2009) Genetic Polymorphism of Inosine Triphosphate Pyrophosphatase Is a Determinant of Mercaptopurine Metabolism and Toxicity During Treatment for Acute Lymphoblastic Leukemia. *Clin Pharmacol Ther* **85:**164-172.

Swann PF, Waters TR, Moulton DC, Xu YZ, Zheng Q, Edwards M, and Mace R (1996) Role of postreplicative DNA mismatch repair in the cytotoxic action of thioguanine. *Science* **273:**1109-1111.

Tay BS, Lilley RM, Murray AW, and Atkinson MR (1969) Inhibition of phosphoribosyl pyrophosphate amidotransferase from Ehrlich ascites-tumour cells by thiopurine nucleotides. *Biochem Pharmacol* **18:**936-938.

Tiede I, Fritz G, Strand S, Poppe D, Dvorsky R, Strand D, Lehr HA, Wirtz S, Becker C, Atreya R, Mudter J, Hildner K, Bartsch B, Holtmann M, Blumberg R, Walczak H, Iven H, Galle PR, Ahmadian MR, and Neurath MF (2003) CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. *J Clin Invest* **111:**1133-1145.

Traut TW (1994) Physiological Concentrations of Purines and Pyrimidines. *Mol Cell Biochem* **140:**1-22.

Vogt MH, Stet EH, De Abreu RA, Bokkerink JP, Lambooy LH, and Trijbels FJ (1993) The importance of methylthio-IMP for methylmercaptopurine ribonucleoside (Me-MPR) cytotoxicity in Molt F4 human malignant T-lymphoblasts. *Biochim Biophys Acta* **1181:**189-194.

Warren DJ, Andersen A, and Slordal L (1995) Quantitation of 6-thioguanine residues in peripheral blood leukocyte DNA obtained from patients receiving 6-mercaptopurine-based maintenance therapy. *Cancer Res* **55:**1670-1674.

Wielinga PR, Reid G, Challa EE, van der Heijden I, van Deemter L, de Haas M, Mol C, Kuil AJ, Groeneveld E, Schuetz JD, Brouwer C, De Abreu RA, Wijnholds J, Beijnen JH, and Borst P (2002) Thiopurine metabolism and identification of the thiopurine metabolites transported by MRP4 and MRP5 overexpressed in human embryonic kidney cells. *Mol Pharmacol* **62:**1321-1331.

Yu J, Batova A, Shao LE, Carrera CJ, and Yu AL (1997) Presence of methylthioadenosine phosphorylase (MTAP) in hematopoietic stem/progenitor cells: Its therapeutic implication for MTAP (-) malignancies. *Clin Cancer Res* **3:**433-438.

Footnotes

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Legends for Figures

Fig. 1 Diagrammatic summary (centre) of pathways of thiopurine metabolism. GMPS, Guanosine monophosphate synthetase EC 6.3.5.2; HPRT, Hypoxanthine-guanine phosphoribosyltransferase EC 2.4.2.8; IMPDH, inosine monophosphate dehydrogenase EC 1.1.1.205; ITPase, Inosine triphosphate pyrophosphatase EC 3.6.1.9. Thionucleotide abbreviations are as used in the text; MeMP, 6-methylmercaptopurine. Structures of thiopurine nucleoside mono-phosphates and the parent drug are shown in red boxes.

Fig.2 LC-MS/MS profiles for thiopurine nucleoside phosphates in standard mixtures and MOLT-4 cell extracts (samples), where X indicates mono-, di- or tri-phosphate. For each plot in A-F, dotted lines represent the monophosphates, solid lines represent the diphosphates and dashed lines represent the triphosphates (Legend in Panel A). A, methyl-thioinosine nucleoside phosphate (meTIXP) standards; B, thioguanosine nucleoside phosphate (TGXP) standards; C, methyl-thioguanosine nucleoside phosphate (meTGXP) standards; D, MOLT-4 cell extract (sample) analysed for meTIXPs; E, MOLT-4 cell extract analysed for TGXPs; F, MOLT-4 cell extract analysed for meTGXPs; in this trace, the identities of additional peaks which were not eluted at the retention times of the standards are unknown. Ordinate scales are arbitrary intensity units.

Fig. 3 Viability curves for MOLT-4 cells treated for 72 h with MP or thiopurine metabolites: A, MP (solid black line, ▲), TGMP (dotted black line, ◆), TGDP (solid grey line, □, grey fill), TGTP (dashed black line, ●); B, meTIMP (dotted black line, ◆), meTIDP (solid grey line, □, grey fill), meTITP (dashed black line, ●), meTGDP (solid black line, ▲); C, TIMP (dotted black line, ◆), TIDP (solid grey line, □, grey fill), TITP (dashed black line, ●).

Fig. 4 Metabolites present in cells after incubation for 72 h with MP or thiopurine nucleotides at the EC_{50} for cell toxicity (Table 1) and measurement by LC-MS/MS. Colour is used to

designate the drug used for cell treatment (specified in the legends), with bars for each drug reflecting the mean (± standard error) concentration of the drug or its metabolites extracted from the cells. A, MOLT4 cells treated with MP or each thioguanosine nucleoside tri-phosphate; B, data in A on an expanded scale to clarify intracellular thioguanosine nucleoside levels; C, a simple nucleotide model of thioguanosine nucleoside phosphate homeostasis. Km values (μM) for Henri-Michaelis-Menten irreversible kinetics were fixed at: guanylate kinase (EC 2.7.4.8: 15), GTPase (100), ATP apyrase (EC 3.6.1.5: 78), guanosine diphosphatase (EC 3.6.1.42: 100). Nucleoside diphosphate kinase activity (EC 2.7.4.6) was modelled with fixed mass action kinetics, setting k (forward and reverse) as 24 min^{-1}. D, fitting rate values (V) to data using the model in C gave V (μM min^{-1}) for guanylate kinase (1578.4), GTPase (3129), ATP apyrase (15), guanosine diphosphatase (8914) and the steady-state ratios of thioguanosine nucleoside phosphates indicated by black bars. A 16-fold increase in V for guanosine diphosphatase was sufficient to reproduce experimental data for TGTP treatment (grey bars).

Fig. 5 Metabolites present in cells after incubation for 72 h with thiopurine nucleoside phosphates at the EC_{50} for cell toxicity (Table 1) and measurement by LC-MS/MS. Colour is used to designate the drug used for cell treatment (specified in the legends), with bars for each drug reflecting the mean (± standard error) concentration of each drug metabolite extracted from the cells. A, MOLT4 cells treated with methyl-thioguanosine nucleoside phosphates (methyl-TGXPs,); B, MOLT4 cells treated with thioinosine nucleoside phosphates (TIXPs where X represents mono-, di- or tri-phosphate); C, MOLT4 cells treated with methyl-thioinosine nucleoside phosphates (methyl-TIXPs).

Fig. 6 Levels of deoxythioguanosine (dTG) incorporated into DNA (moles dTG per 1000 moles of deoxyadenosine [dA]) of MOLT4 cells after culture with equitoxic doses of thiopurine metabolites. Metabolites were used at their EC_{50} concentrations (Table 1) and the cells cultured for 72 h before extraction and analysis. Bars are arranged, left to right in order of decreasing cytotoxicity as given in Table 1. TIDP and TITP were not included in these experiments as they would be expected to contribute only by dephosphorylation to TIMP. Values were compared by ANOVA to the control (MOLT4 cells without thiopurine; c) with Dunnett's post-hoc test; error bars were derived from ANOVA and statistical significance compared to the background control (no thiopurine treatment) is indicated by: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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TABLES

Table 1

Effects of individual metabolites on MOLT-4 cell viability. Data are listed in order of decreasing cytotoxicity.

Drug	EC_{50} (μM)	SE
meTIMP	0.185	0.026[m]
TGTP	0.265	0.076[§]
TIMP	0.345	0.055[m]
TGDP	0.360	0.104[m]
meTIDP	0.393	0.085[m]
meTITP	0.396	0.039[§]
TIDP	0.412	0.097[m]
TGMP	0.619	0.051[m]
TITP	0.956	0.214[m]
MP	2.92	0.415[m]
meTGTP	15.37	3.556[m]
meTGDP	>2000	ND
meTGMP	>2000	ND

Model fitted: [m]mixed-effects; [§]mean from separate curves

ND, compounds not toxic and EC_{50} could not be estimated

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Table 2

Contributions (%) of variables to principal components (PC)

Variable	PC1	PC2	PC3	PC4
DNA	24.9	13.6	56.77	4.7
TGXP	32.02	2.9	5.55	59.5
meTGXP	30.05	0.76	33.43	35.76
meTIXP	13.04	82.7	4.25	1.97

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Fig. 1

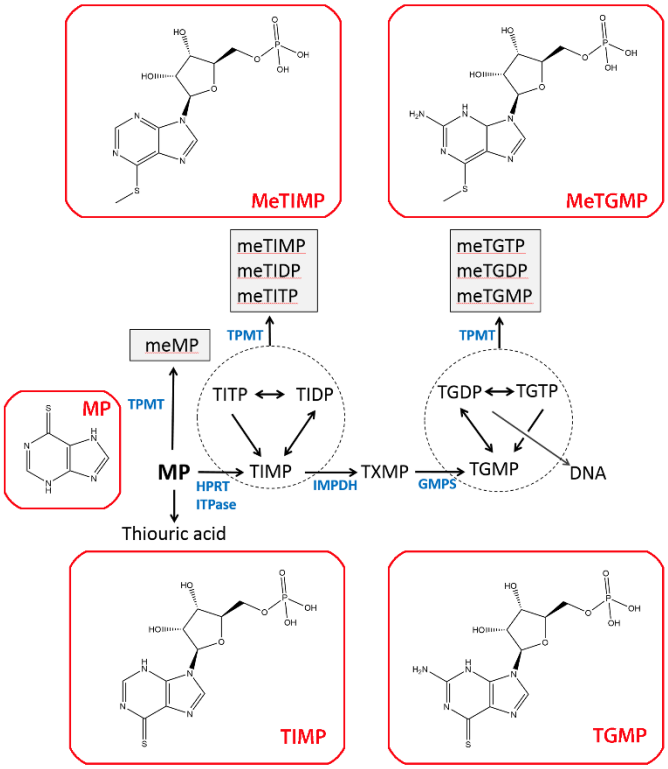

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Fig. 2 LC-MS/MS chromatograms: standards and examples

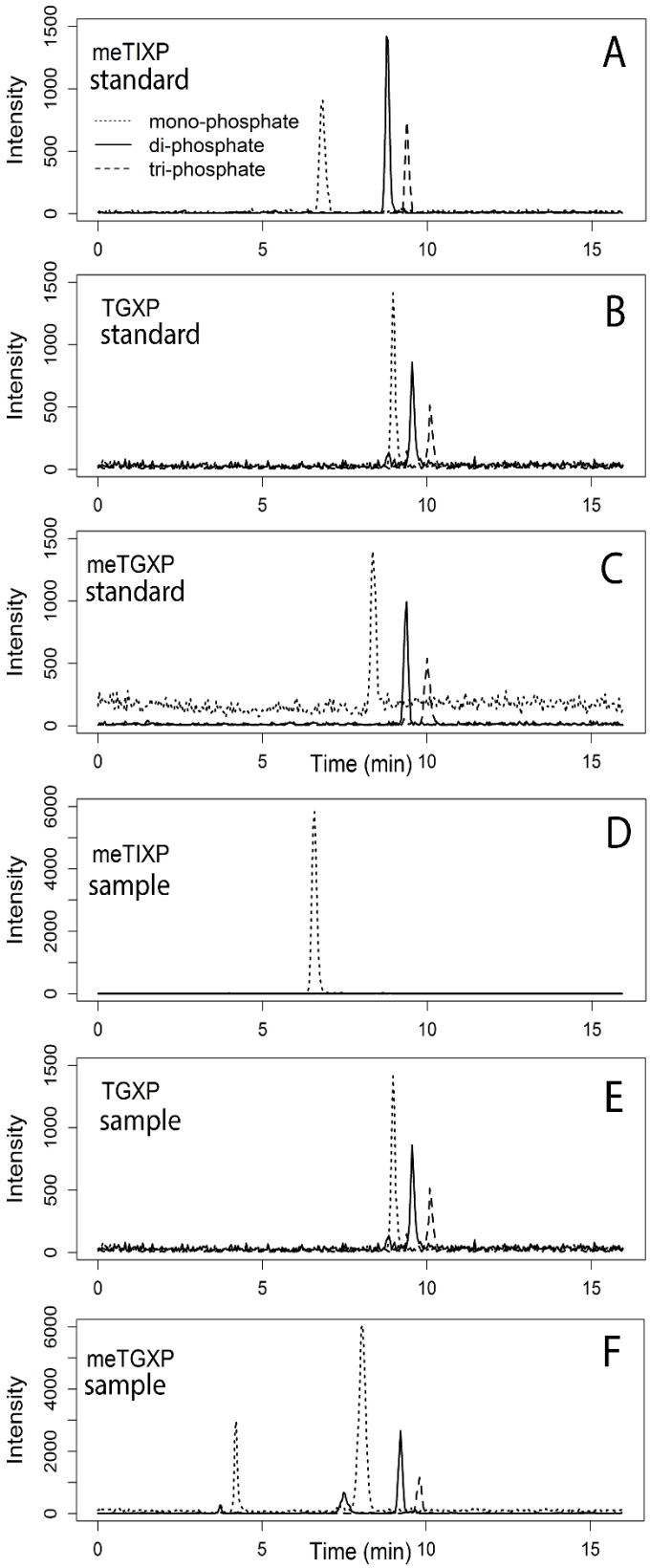

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Fig 3

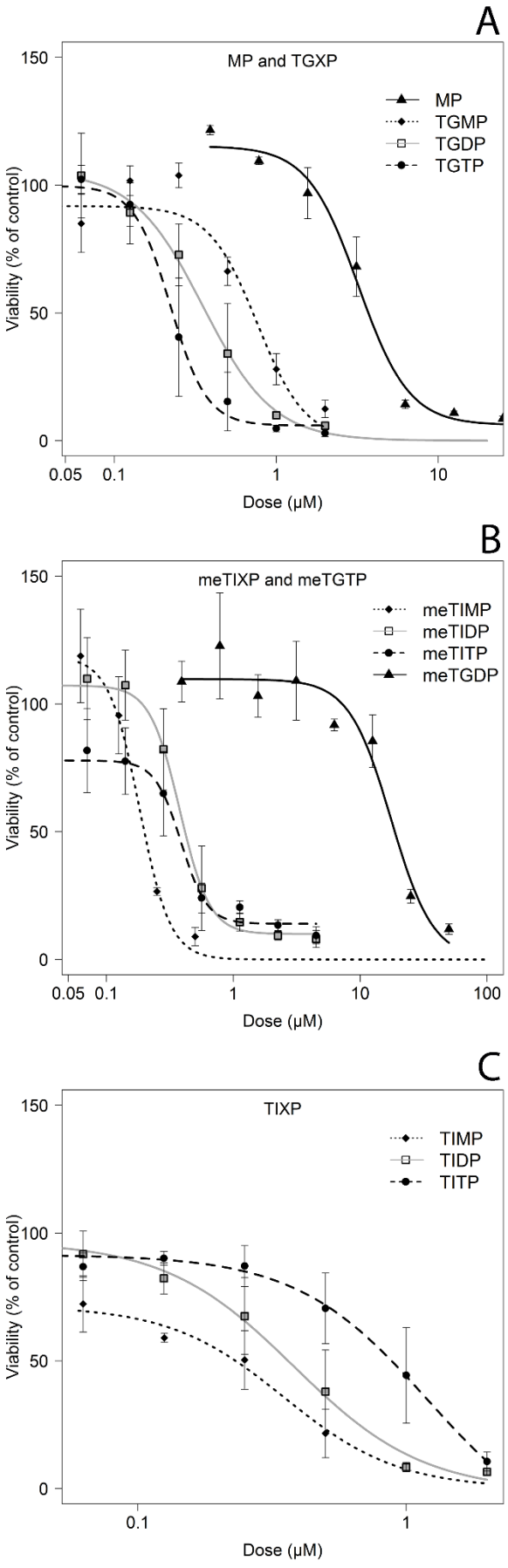

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Fig. 4

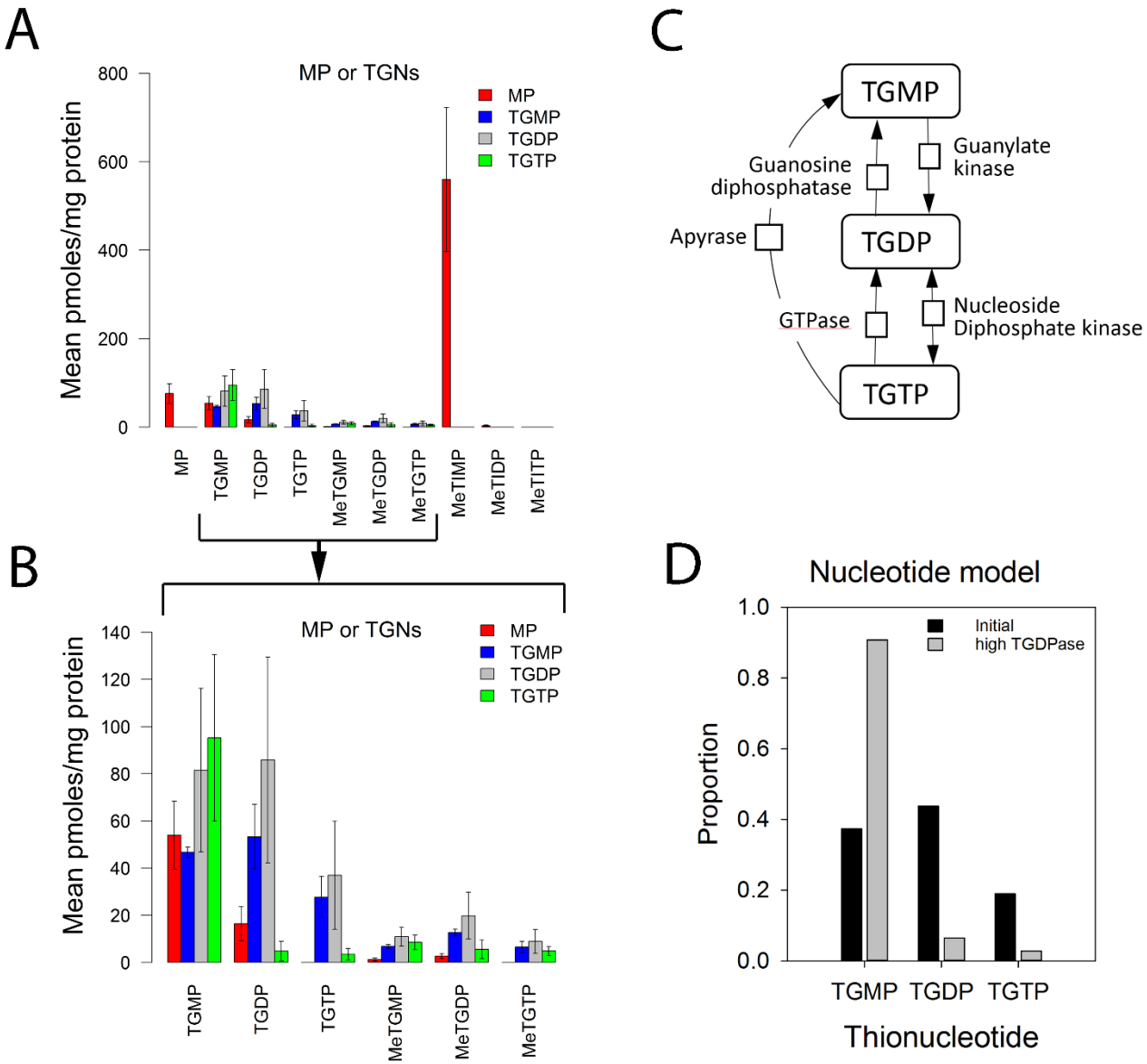

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Fig. 5

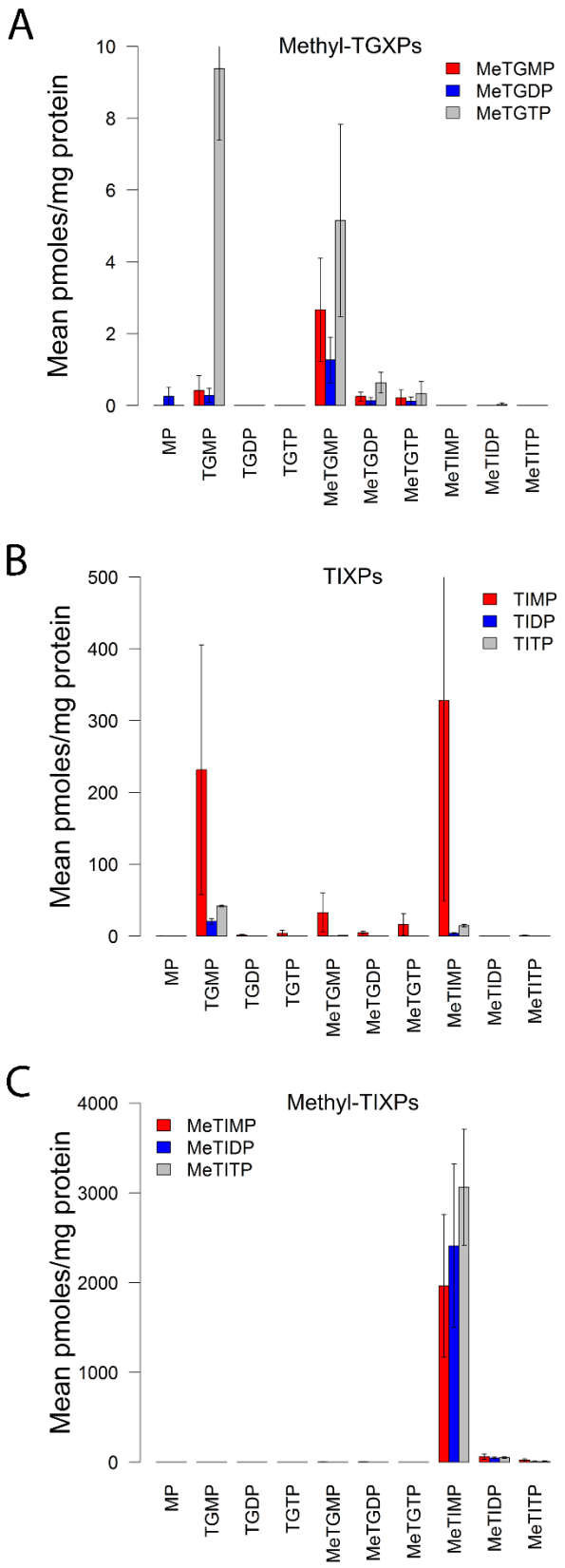

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Fig. 6