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Short communication

Liquid chromatography–mass spectrometry for measuring deoxythioguanosine in DNA from thiopurine-treated patients

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ABSTRACT

Adverse reactions and non-response are common in patients treated with thiopurine drugs. Current monitoring of drug metabolite levels for guiding treatment are limited to analysis of thioguanine nucleotides (TGNs) in erythrocytes after chemical derivatisation. Erythrocytes are not the target tissue and TGN levels show poor correlations with clinical response. We have developed a sensitive assay to quantify deoxythioguanosine (dTG) without derivatisation in the DNA of nucleated blood cells. Using liquid chromatography and detection by tandem mass spectrometry, an intra- and inter-assay variability below 7.8% and 17.0% respectively were achieved. The assay had a detection limit of 0.0003125 ng (1.1 femtomoles) dTG and was quantified in DNA samples relative to endogenous deoxyadenosine (dA) in a small group of 20 patients with inflammatory bowel disease, all of whom had been established on azathioprine (AZA) therapy for more than 25 weeks. These patients had dTG levels of 20–1360 mol dTG/10^6 mol dA; three patients who had not started therapy had no detectable dTG. This method, comparable to previous methods in sensitivity, enables the direct detection of a cytotoxic thiopurine metabolite without derivatisation in an easily obtainable, stable sample and will facilitate a better understanding of the mechanisms of action of these inexpensive yet effective drugs.

1. Introduction

Since their introduction into clinical practice more than six decades ago, the purine analogues mercaptopurine (MP; 3,7-dihydropurine-6-thione), azathioprine (AZA; 6-[3-methyl-5-nitroimidazol-4-yl]sulfanyl-7H-purine) and thioguanine (TG; 2-amino-3,7-dihydropurine-6-thione) have been used extensively in the treatment of diseases such as acute childhood leukaemia (ALL) [1], inflammatory bowel disease (IBD) [2,3], auto-immune hepatitis [4] and rheumatoid arthritis [5]. Thiopurines require activation by hypoxanthine-guanine phosphoribosyl transferase (HGPRT, E.C. 2.4.2.8) followed by multi-step metabolism to TGNs or methylated products to exert their clinical effect (Fig. 1). Two key enzymes mediating thiopurine metabolism are inosine-monophosphate dehydrogenase (IMPDH) which is only present in nucleated cells [6], and thiopurine methyltransferase (TPMT) [7–9]. If treated with normal thiopurine doses, patients lacking TPMT activity develop high TGN levels that can lead to life-threatening leukopenia [10–12]. Cytotoxicity is mediated by a variety of mechanisms, including inhibition of *de novo* purine synthesis, disruption of G-protein signalling [13] and incorporation of thioguanine nucleotides (TGNs) into DNA with subsequent mismatching to thymidine, causing cell death by post-replicative mismatch repair [14–16].

Given a low therapeutic index and the wide variation in clinical response, including potential for life-threatening toxicity in patients with very low or absent TPMT activity, it is important to monitor and optimise thiopurine drug levels. Assays have been

Abbreviations: ALL, Acute childhood leukemia; 6-MP or MP, 6-mercaptopurine; AZA, azathioprine; IBD, inflammatory bowel disease; MeMP, methylmercaptopurine; MeMP-d3, Methymercaptopurine-D3; TIMP, thioinosine monophosphate; TIDP, thioinosine diphosphate; TITP, thioinosine triphosphate; TXMP, thioxanthine monophosphate; TGMP, thioguanosine monophosphate; TGDP, thioguanosine diphosphate; TGTP, thioguanosine triphosphate; IMPDH, inosine-monophosphate dehydrogenase; TPMT, thiopurine methyl-transferase; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; ITPase, inosine tri-phosphatase; GMPS, guanosine monophosphate synthetase; dTG, deoxythioguanosine; TGN, thioguanine nucleotide; NBCs, nucleated blood cells; CD, Crohn's disease; UC, ulcerative colitis; RBC, red blood cell; QC, Quality control.

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Table 1
Mass transitions and optimised MS/MS parameters for analyte quantification, deoxythioguanosine (dTG), deoxyadenosine (dA), Methymercaptopurine-D3 (MeMP-d3).

Analyte	Analyte Retention Time (min)	MRM transition (*m*/*z*)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (eV)	Collision exit potential (V)
dTG	6	284.19 –> 168.2	41	10	15	10
dA	5.6	252.30 –> 136.2	31	10	21	8
MeMP-d3	6.9	170.1 –> 152.2	66	10	33	10

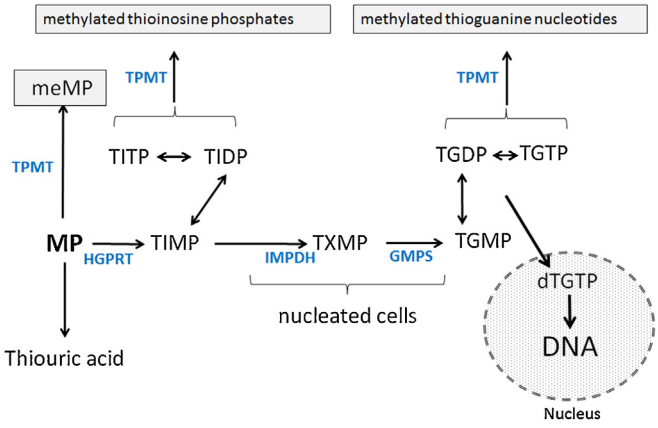

Fig. 1. Schematic summarising the metabolism of 6- MP via the enzymes (Blue text) inosine-monophosphate dehydrogenase (IMPDH), thiopurine methyl-transferase (TPMT), hypoxanthine-guanine phosphoribosyl transferase (HGPRT), inosine triphosphatase (ITPase) and guanosine monophosphate synthetase (GMPS). MeMP, methylmercaptopurine; TIMP, thioinosine monophosphate with metabolism to the di- and triphosphates TIDP and TITP, respectively; TXMP, thioxanthine monophosphate; TGMP, thioguanosine monophosphate with metabolism to the di- and tri-phosphates TGDP and TGTP, respectively and incorporation into DNA (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

developed to measure metabolites in a range of cellular compartments including erythrocytes [17–20], whole blood [21] and leukocyte DNA [22,23] but ease of access to erythrocytes coupled with simple HPLC separation techniques has meant that quantifying thiopurine metabolites in erythrocytes has become the standard method for therapeutic monitoring. However, despite its value for assessing patient compliance, there is significant debate about concordance with therapeutic response in IBD [24–26]. Non-concordance can arise from methodological issues [27] and differences in metabolism between nucleated versus enucleated cells which lack the critical IMPDH enzyme. Therefore, the incorporation of deoxythioguanosine (dTG) into the DNA of nucleated cells may be a more relevant marker of therapeutic response. The aim of this study was to develop a sensitive assay for DNA-incorporated dTG in nucleated blood cells which could be developed for clinical use to study the mechanisms of response to thiopurines.

2. Materials and methods

2.1. Chemicals and enzymes

The dTG standard was from Carbosynth (Compton, UK); deoxyadenosine (dA) was from Sigma-Aldrich (Gillingham, UK) and deuterated 6-methylmercaptopurine (MeMP-d3) from Toronto Research Chemicals (Ontario, Canada). HPLC grade acetic acid was from Fisher Scientific (Loughborough, UK). Calf intestinal alkaline phosphatase and nuclease P1 from *Penicillium citrinum* were from Sigma-Aldrich, as were all other reagents.

2.2. Patient blood sample collection and processing

Clinical samples were from a small cohort of adult IBD patients (10 with Crohn's Disease [CD] and 10 Ulcerative Colitis [UC]) treated with a range of doses of AZA, all of whom had been in clinical remission for more than 6 months with no therapeutic complications; three untreated IBD patients were used as controls. The study protocol was approved by NRES Committee South West – Cornwall & Plymouth, Bristol Research Ethics Committee Centre. DNA was isolated from whole blood collected in EDTA tubes, or from negative-control MOLT4 (T-acute lymphoblastic leukaemia) cells, using previously published methods [28]. Briefly whole blood was mixed with 3 vols of ice-cold buffer A (10 mM Tris, 320 mM sucrose, 5 mM Mg Cl_2 1% Triton × 100 pH 8) and centrifuged at 1730*g* for 10 min at 4 °C. The supernatant was removed and the remaining pellet re-suspended in 1 mL buffer B (400 mM Tris, 60 mM EDTA, 150 mM NaCl and 1% SDS pH 8) plus 0.5 mL of 5 M sodium perchlorate, mixed for 10 min, then incubated at 65 °C for 45 min. To this, 2.5 mL of chloroform was added and mixed for 20 min prior to centrifugation at 432*g* for 10 min at 4 °C. The top layer was removed and 2.5 vols of ethanol added to precipitate the DNA which was spooled out, air-dried and re-suspended in 100–200 μL of double-deionised water. The red blood cell (RBC) TGN assays were performed by a commercial laboratory at the City Hospital Birmingham (cityassays.org). Other tests were part of routine clinical care at the six hospitals contributing samples.

DNA was digested with P1 nuclease and alkaline phosphatase to release nucleosides for LC–MS/MS analysis using previously-described methods [29]. Briefly, samples were prepared in the following manner: 5 μg DNA in a total volume of 100 μL double-deionised water, containing 124.38 ng/mL (0.735 μM) Methymercaptopurine-D3 (MeMP-d3) as an internal standard to control for extraction efficiency, was denatured by heating to 100 °C for 5 min. After chilling on ice for 2 min, 10 μL of 10X digestion buffer (500 mM sodium acetate, 10 mM $MgCl_2$ pH 5.3) and 5 μL of 0.12U/μL nuclease P1 was added and incubated for 1 h at 50 °C. Finally, 20 μL of 1 M Tris-HCl and 1 μL of alkaline phosphatase (1U/mL) were added to each sample and incubated for 30 min at 40 °C. MeMP-d3 was used as an internal standard as it was the only deuterated thiopurine metabolite available commercially at the time.

2.3. LC–MS/MS analysis of thioguanine incorporated into DNA

Chromatographic separation of dTG, dA and MeMP-d3 was achieved using a Prominence HPLC (Shimadzu, Kyoto, Japan) with an XSelect HSS T3 4.6 × 100 mm 3.5 μm and a VanGuard cartridge 3.9 × 5 mm 3.5 μm guard column (Waters, Massachusetts, USA) maintained at 30 °C. Analytes were eluted with HPLC grade (Sigma-Aldrich) mobile phases comprising 0.05% aqueous formic acid (A) and 0.05% formic acid in acetonitrile (B). The flow rate was 0.5 mL/min and the mobile phase system consisted of a starting condition of 1% buffer B increasing to 3% at 1.1 min, 8% at 2.4 min and increasing to a maximum of 30% at 4.1 min then decreasing to 5% at 4.5 min, maintained until 5.5 min then decreasing to 1% for an equilibration period of 2.5 min. An API4000 triple quadrupole LC–MS/MS (Applied Biosystems, California, USA) was used for analysis with electrospray ionisation performed in positive ion mode using nitrogen gas with the following optimum settings: curtain gas, 20; ion source gas 1, 10; ion source gas 2, 10; ion spray voltage, 5500; collision gas, 6; entrance potential, 10; ionisation tempera-