Supplementary Information for

Pristup et al., Molybdenum cofactor catabolism unravels the physiological role of the drug metabolizing enzyme thiopurine S-methyltransferase

Supplementary Information Text

Supplemental methods

Preparation of organ extracts

Mouse brain, liver and kidney raw lysates were prepared by resuspension and homogenization in 0.1 M Tris/HCl, pH 7.5 containing protease inhibitor (Roche). Samples were centrifuged three times at 21,000 x *g* for 20 min at 4°C. Lysate samples were shock frozen in liquid nitrogen and stored at -80°C For preparation of human liver cytosol samples 1 g of human liver samples were homogenized in 10 mM HEPES containing 1 mM EDTA, 0.15 mM KCl, 1 mM DTT and 200 µM protease inhibitor (Pefabloc, Roth). Cytosolic fractions were collected after differential centrifugation and stored at –80°C. For human erythrocyte cytosolic lysates (RBC) EDTA-Blood samples were centrifuged for 10 min at 3000 rpm and washed once with 0.9% NaCl. The pellet was resuspended in 5x-volume of 20 mM potassium phosphate buffer (pH 7.4) and stored at -80°C.

Cloning, expression and purification of recombinant human TPMT

The coding sequence of human *TPMT* (NM_000367.2) was PCR-cloned into pQE80L (Qiagen) using BamHI and Xmal restriction sites. The identity of the generated construct was verified by DNA sequencing (GATC). TPMT protein was expressed in *E. coli* BL21 (DE3) strain. Expression was induced using 0.25 mM isopropyl ß-thiogalactoside at A_{600} of 0.6 and continued for 15 h at 24°C. The N-terminally His-tagged TPMT was purified by nickel nitrolotriacetic acid affinity (Macherey-Nagel). Purified protein was buffer exchanged (50 mM Tris/HCl, pH 7.5) and stored at –80°C. The identity of the TPMT was confirmed by western blot using the TPMT-specific rabbit polyclonal anti-TPMT antibodies (H-78) (Santa Cruz Biotechnology).

Quantification of urothione by LC-MS-MS

Synthesis of internal standard [$^{13}C^{2}H_3$]urothione

Urothione was isolated from human urine by chromatographic separation on Porapak Rxn RP (5g) cartridges. The crude product was purified by low pressure liquid chromatography (CombiFlash Rf$^+$, Axel Semrau, Sprockhövel, Germany) on a Lobar Lichroprep RP18 column (310 mm x 25 mm i.d., 40-63 µm, Merck Chemicals, Schwalbach, Germany) at a flow rate of 10 ml/min using a gradient of methanol and water from 5:95 v/v to 20:80 v/v. The fraction containing urothione was evaporated to dryness and the residue dissolved in 0.25 M sodium carbonate in water at 70 °C. Urothione was precipitated by addition of glacial acetic acid.

[$^{13}C^{2}H_3$]urothion was obtained by S-demethylation of urothione with sodium in liquid ammonia according to Lutgring et al. [1] and subsequent methylation with [$^{13}C^{2}H_3$]methyl iodide as described for the synthesis of [$^{2}H_3$]6-methylthioinosine 5'-phosphates [2]. Purification was performed as described for unlabelled urothione. Composition and purity of urothione and [$^{13}C^{2}H_3$]urothione was checked by LC-MS/MS analysis and HPLC-UV analysis. The internal standard contained less than 0.05 % of unlabelled urothione.

Sample preparation

To 25 µl of urine, 10 µl of internal standard [$^{13}C^{2}H_3$]urothione (0.25 pmol/µl in mobile phase; water:methanol 95:5 v/v with 0.1 % formic acid) and 15 µl of mobile phase were added. Mouse urine samples (5-25 µl) were diluted to a total volume of 40 µl with mobile phase and spiked with 10 µl of internal standard. Samples were vortex mixed, and 2 µl were used for LC-MS-MS analysis. For urothione analysis in kidney samples, 100 µL of the aqueous extract from the non-targeted metabolomics experiment was spiked with 10 µL internal standard, dried at 40°C under nitrogen and reconstituted in 30 µL methanol: water (0.5:9.5, v/v) containing 0.1% formic acid. 10µL was used for LC- MS-MS analyses.

LC-MS-MS analysis

An Agilent 6460 triple quadrupole mass spectrometer (Agilent, Waldbronn, Germany) coupled to an Agilent 1200/1290 HPLC system consisting of binary pump G4220B, well-plate sampler G1367D and column thermostat G1316B was used. Ionization mode was electrospray (ESI), polarity positive. Electrospray jetstream conditions were as follows: capillary voltage 4000 V, nozzle voltage 1000 V, drying gas flow 10 l/min nitrogen, drying gas temperature 325°C, nebulizer pressure 50 psi, sheath gas temperature 350 °C, sheath gas flow 10 l/min. HPLC separation was achieved on a Poroshell 120 EC-C18 column (50•2.1 mm, 2.7 µm particle size, Agilent, Waldbronn, Germany) using (A) 0.1 % formic acid in water, and (B) 0.1 % formic acid in methanol as mobile phases at a flow rate of 0.6 ml/min. Gradient started at 5 % B from 0 min to 0.5 min, linear increase to 10 % B to 0.75 min, linear increase to 13 % B to 2.5 min, linear increase to 100 % B to 3 min, remaining at 100 % B to 4 min, then re-equilibration. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode, using the respective

$[M+H]^+$ ions as precursor ions, *m/z* 326.0 and 330.0 for urothione and $[^{13}C^2H_3]$urothione, and the product ion *m/z* 233.1 for both compounds. Dwell time was 100 ms, fragmentor voltage was set at 105, and the collision energy at 25 for urothione and 29 for the internal standard.

Standardization and validation

Calibration samples were prepared in mobile phase in the concentration range from 0.625 pmol/ml to 1250 pmol/ml, worked up as described above, and analyzed together with the unknown samples. Calibration curves based on internal standard calibration were obtained by weighted (1/x) linear regression for the peak-area ratio of the analyte to the internal standard against the amount of the analyte. The concentration of the analytes in unknown samples was obtained from the regression line. Assay accuracy and precision were determined by analyzing quality controls that were prepared like the calibration samples.

Normalisation of urinary urothione levels in humans

Urinary urothione levels were normalised to levels of creatinine, Creatinine was determined in the clinical laboratory of the Robert-Bosch Hospital (Stuttgart, Germany) with a VITROS system (Ortho-Clinical Diagnostics, Neckargemünd, Germany).

Non-targeted metabolomics

Kidney samples (16-44 mg, derived from 4 wild-type and 4 *Tpmt*$^{-/-}$ mice) were subjected to tissue homogenization and metabolite extraction as described [3,4]. Briefly, aqueous compounds were recovered in 1:1 (v/v) methanol/water (aqueous extract) by a bead-beating approach (40 µL solvent/mg tissue). After centrifugation, the supernatant was separated and organic compounds were recovered by consecutive extraction of the pellet with 1:3 (v/v) methanol/methyl *tert*-butyl ether (organic extract) using the same solvent to tissue ratio as described for the aqueous extract. Extracts were dried at 40°C under nitrogen and stored at -20°C until analysis. Aqueous extracts were reconstituted in acetonitrile: water (9.5:0.5, v/v) and organic extracts were resuspended in isopropanol: methanol (3:1, v/v) prior separation by hydrophilic liquid interaction chromatography (aqueous extracts) and reversed phase chromatography (organic extracts). Metabolites were analyzed by quadrupole time-of-flight mass spectrometry (6550 iFunnel Q-TOF LC/MS, Agilent Technologies) in negative and positive ionization mode, respectively. Quality control samples (QCs) were prepared as pooled aliquots from each extract and analyzed throughout the analytical batch for column conditioning and to assess the analytical performance. Mass spectral data processing and statistical analysis was carried out with Agilent Mass Hunter Qualitative Analysis (version B.06.00), Profinder (version B.06.00) and Mass Profiler Professional (version 13) software tools.

Supplemental Table S1

Demographic data of the individuals (n= 213) of the population-based Study of Health in Pomerania (SHIP) cohort

	Study cohort (n=213)
sex, n (%)	
male	108 (50.7)
female	105 (49.3)
age [median (range)], years	52 (22-82)
waist size [median (range)], cm	93 (59-150)
BMI [median (range)], kg/m²	28 (18-48)
myocardial infarction, n (%)	
yes	6 (2.8)
no	207 (97.2)
stroke, n (%)	
yes	5 (2.3)
no	208 (97.7)
diabetes type 2, n (%)	
yes	30 (14.1)
no	183 (85.9)
smoking habit, n (%)	
ex-smoker	88 (41.3)
current smoker	56 (26.3)
never smoker	69 (32.4)

Supplemental Figures

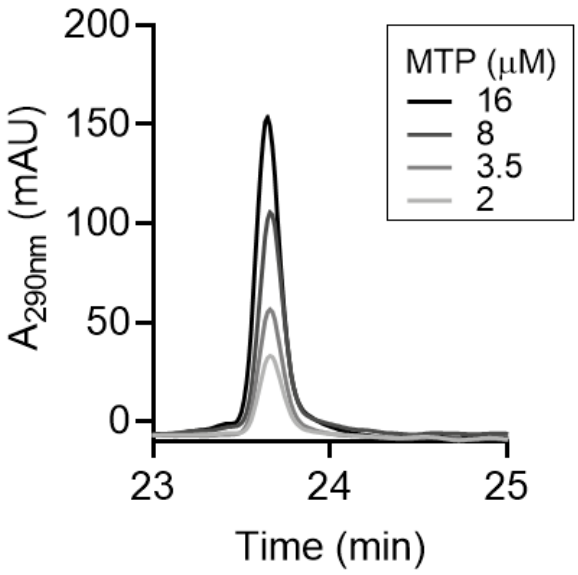

Figure S1. TPMT-dependent methylation of thiopterin. HPLC C18 chromatograms of the detection of urothione (t_R=23.7 min). TPMT (20 ng/µl) was coincubated with 2-16 µM sulfite oxidase Mo-domain (non-denaturated, molybdopterin [MPT]) in the presence of 100 µM SAM. Subsequent treatment with AP was applied to convert all reaction product into urothione.

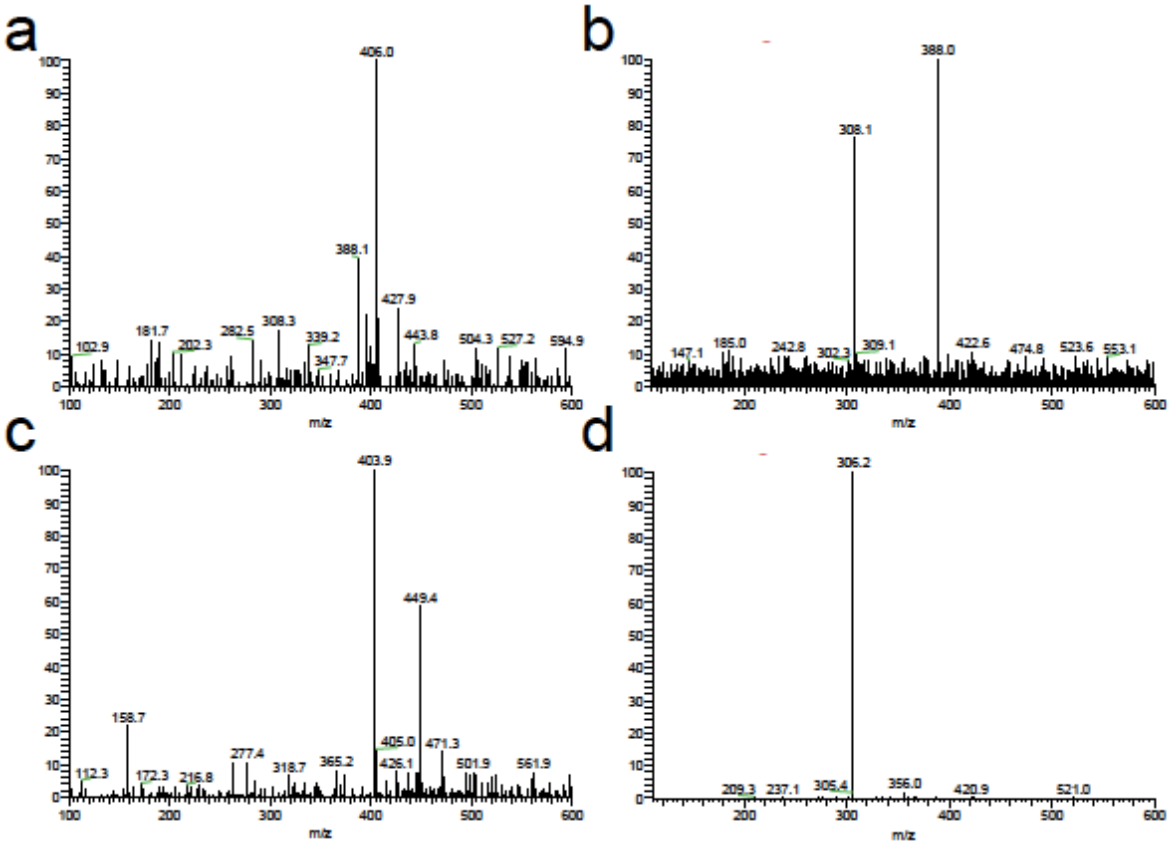

Figure S2. HPLC-MS analysis of the TPMT product in the positive (a, b) and negative (c, d) ion mode. a. MS spectrum of the TPMT product in the positive mode showing a molecular ion peak at 406 m/z**. b.** MS/MS-spectrum of the TPMT product shown in panel (a) (406 m/z). The two major fragmented ion peaks at 308 m/z and at 388 m/z can be explained by the loss of a H_3PO_4 (ΔM= 98 m/z) and H_2O (ΔM=18 m/z). **c.** MS spectrum of the TPMT product in the negative mode showing a molecular ion peak at 404 m/z**. d.** MS/MS spectrum of the TPMT product shown in panel (c) (404 m/z). The fragmented ion peak at 306 m/z can be explained by the loss of a H_3PO_4 (ΔM= 98 m/z).

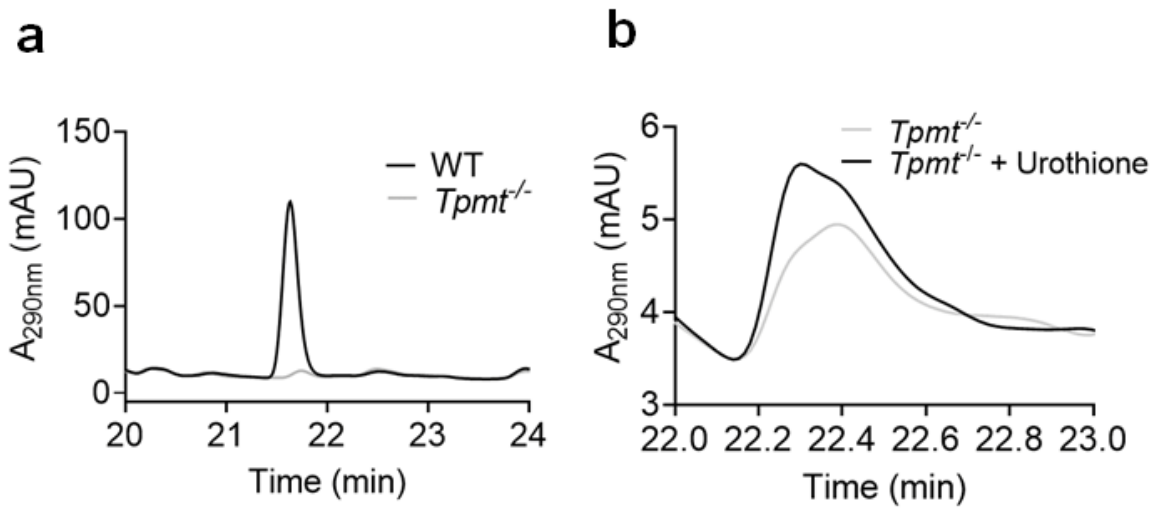

Figure S3. HPLC analysis of *in vitro* urothione synthesis using *Tpmt*$^{-/-}$ and wildtype mice liver extracts. a. HPLC C18 chromatograms of urothione (t_R=22.3 min) in vitro synthesized by crude liver extracts from *Tpmt*$^{-/-}$ and wildtype (WT) mice. 80 µL of each reaction mixture were injected. **b.** Zoom in of the HPLC chromatogram surrounding the elution time of urothione. The sample derived from a *Tpmt*$^{-/-}$ mouse crude extract (40 µL) was re-run after the addition of 17 pmol urothione and shows a change in the shape of the peak indicating a different between the elution of in sample urothione and the unknown substance present in the *Tpmt*$^{-/-}$ extract.

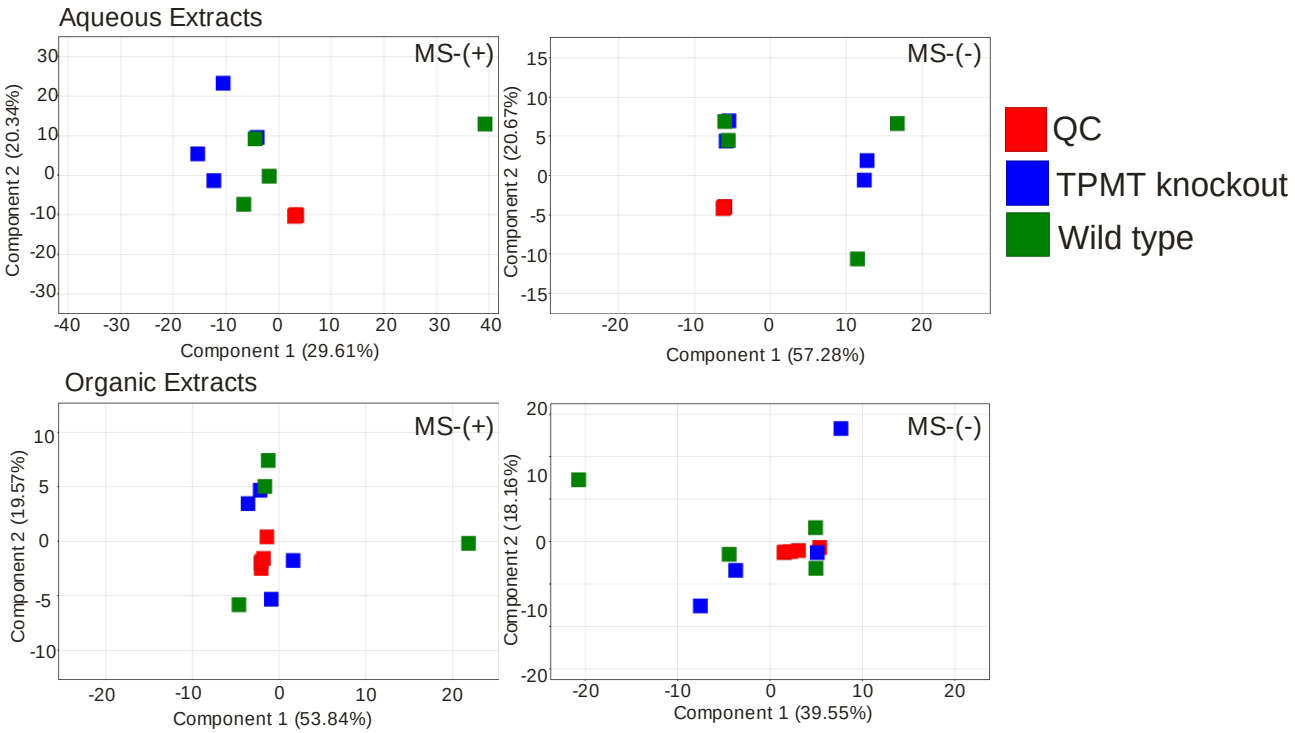

Figure S4. Untargeted metabolomics in kidney tissue of mice. Principle component analysis of QC samples (red), *Tpmt*$^{-/-}$ (blue) and wild type mice (green) in aqueous extracts and organic extracts in positive and negative electrospray ionization mode. In the principle component analysis (PCA) of the QC sample, TPMT knockout mice and the wild type mice is shown in the aqueous and organic extract in positive and negative electrospray ionization mode. The QC samples cluster in each analytical mode together, which indicates robust and reproducible analytical performance. None of the PCA shows a separation between the *Tpmt*$^{-/-}$ and wild type mice. Moreover by means of univariate data analysis for each analytical mode, no relevantly and significantly regulated features could be found (absolute log2 fold change ≥ 1 and unpaired t-test with Benjamini-& Hochberg [5] adjusted $p \leq 0.05$).

SI References

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3. García-Cañaveras, J. C., Donato, M. T., Castell, J. V. & Lahoz, A. A comprehensive untargeted metabonomic analysis of human steatotic liver tissue by RP and HILIC chromatography coupled to mass spectrometry reveals important metabolic alterations. *Journal of proteome research* **10**, 4825–4834 (2011).
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