ADDENDUM

Colonic thioguanine pro-drug: Investigation of microbiome and novel host metabolism

Timothy Florina, Ramya Movva1, Jakob Beguna, John Duleyc, Iulia Oanceaa, and Páraic Ó. Cuíd

1Mater Research – University of Queensland, Translational Research Institute, Queensland, Australia; 2Mater Research – University of Queensland, Queensland, Australia; 3Pharmacy Australia Centre of Excellence, University of Queensland, Queensland, Australia; 4Diamantina Institute – University of Queensland, Translational Research Institute, Queensland, Australia

ABSTRACT

Thiopurines are analogues of endogenous purines. They are pro-drugs which require the purine salvage pathway to convert them to the active drug nucleotides (TGN). These drugs are used to maintain clinical remission in patients with inflammatory bowel diseases. In our recent Gut paper, we showed that thioguanine worked quickly to improve colitis in the absence in the host animal of the key guanine salvage enzyme, hypoxanthine-guanine-phosphoribosyltransferase (HPRT). Current evidence favours the proposition that active drug delivery to the host lacking HPRT requires translocation of TGN-loaded bacteria across the inflamed mucosal barrier, and most likely delivery by phagocytosis. Alternatively, the efficacy of thioguanine in treating colitis could be mediated by modulation of the community of the microbiota in the intestine, or there are novel host pathways for conversion of the thioguanine pro-drug to TGN.

KEYWORDS

colitis; Harnessing microbial strategies for treatment of human disease; microbiome; phagocytosis; purine salvage; Role of commensal flora in GI diseases; Role of gut microbiome in GI disease; thioguanine

Thioguanine (TG), a thiopurine pro-drug, is an analogue of guanine. It has been shown to be effective in open-label studies in both Crohn’s disease and ulcerative colitis (UC).1-4 TG is well tolerated but it is rarely used for fear of liver vascular toxicity resulting in portal hypertension.5,6 We previously showed that the liver vascular toxicity is dose-related arising from excessive thioguanine nucleotide triphosphate (TGTP) or related metabolites in the portal circulation’s sinusoidal endothelium.7 Notably the liver vascular toxicity is not due to cumulative drug in the liver, and it can be avoided by utilizing low once daily doses of TG8 or splitting the daily dose.4 Both dosing approaches reduce the peak concentration (Cmax) of TG in the portal circulation. However, once daily low-dosing is less likely to result in effective immunomodulation.7

In our preclinical mouse model of UC, TG has major advantages over mercaptopurine (MP), which is a more commonly used thiopurine pro-drug. In our paper published in Gut this year,9 and accompanied by an editorial from one of the leaders in the field of inflammatory bowel diseases, Neurath,10 we showed that –

• oral TG treatment acts faster than MP,
• rectal TG improved murine colitis locally within 7 days. MP is an analogue of the endogenous purine base, hypoxanthine, and the superior efficacy of TG is likely because the conversion of MP to TGTP is rate-limited by the purine enzyme, inosine monophosphate dehydrogenase, and the colonic dwell time is not sufficiently long.
• myelosuppression and liver toxicity, significant problems with oral TG,7 are avoided with colonic delivery.9 This is presumably because colonic absorption is less than small intestinal absorption.

TG and microbiota in colitis

In our Gut paper,9 we concluded that colonic microbial metabolism of TG was a significant pathway to deliver TGTP locally to the inflamed gut mucosa. This is the opposite of the usual scenario of drug
disposition, where the luminal colonic metabolism is considered to be a metabolic sink that competes with host pro-drug metabolism. This conclusion was supported by the surprising observation that oral TG improved DSS-induced colitis in Hprt (hypoxanthine-guanine phosphoribosyl transferase)-deficient (Hprt-ko) mice. HPRT is the highly conserved purine salvage enzyme. Oral TG was not associated with myelosuppression in Hprt-ko mice. Furthermore, we showed that representative axenic gut bacterial and faecal cultures convert TG to thioguanine nucleotides. Following TG gavage, thioguanine nucleotides were detected in wild type (WT) and Hprt-ko mouse faecal bacteria and in WT liver but not in Hprt-ko mouse liver.

We have since confirmed that gavaged TG improves spontaneous Winnie colitis (vide infra) in the absence of Hprt, by crossing Winnie with Hprt-ko (HaW) mice. Similar to Winnie, HaW have a pancolitis which is worse distally, and oral TG treatment results in a reduction in diarrhoea compared to gavage controls (Fig. 1, left panel) and an improvement in blinded histological colitis scores (Fig. 1, right panel showing distal colon scores only). Notably, Winnie but not HaW mice gavaged with 1.5 mg/kg/d TG for 14 days become immunosuppressed (Winnie mean white blood cell (WBC) 2.2 v 11.7 \times 10^9/L, P <0.01; HaW mean WBC 8.8 v 11.8 \times 10^9/L, P 0.3).

We did not investigate in our Gut paper how TGTP transfers from the bacteria to the host. TGTP does not exist extracellularly in mammals and we have not detected thioguanine nucleotides in faecal water. Host transfer of TG would therefore require translocation of TGN-loaded bacteria across the mucosal barrier, and most likely delivery by phagocytosis. This would theoretically deliver the active drug to the inflamed mucosa. We have both published and unpublished evidence to support the proposition that bacteria could deliver TGN in this way and that their therapeutic efficacy may be mediated by modulating autophagy pathways.

- We showed in our Gut paper that TG increased the efficiency of bulk autophagy by both epithelial and macrophage cells.
- We also showed that TG increased bacterial elimination via autophagy in Salmonella-infected epithelial cells.
- In pilot unpublished in vitro studies, we found that bacterial autophagy was augmented in Salmonella-infected colonic epithelial cells, which had been pre-incubated for 120 minutes with TG 12.5–50 \mu M (Fig. 2).

Alternatively, the efficacy of TG treatment may be mediated by modulation of the colonic microbiota. Using bacterial 16S-rRNA profiling we demonstrated that TG exerts a biotic effect on the microbiota of wild-type (WT) mice. However we failed to find evidence for this in our spontaneous Winnie colitis.

**Figure 1.** Oral thioguanine improves spontaneous colitis in the absence of host Hprt. Left panel. HaW mice (blue) have less diarrhoea (mean + SD) with TG gavage for 14 d. Right panel. Winnie (green) and HaW blinded distal colitis scores (mean + SE). Mann-Whitney non-parametric test. *P < 0.05, **P < 0.001.

**Figure 2.** Autophagy was augmented by preloading Salmonella with thioguanine for 120’. The proportion bacteria in LC3+ autophagosomes was augmented with 12.5-50\mu M TG after infecting LS174T cells for 30’. *P < 0.05 Mann-Whitney non-parametric test.
model. Nonetheless, it remains a possibility that the microbiota’s functional activity (as opposed to its genomic potential) is less colitogenic with TG treatment.

We are more fully investigating these possibilities in our Winnie and induced dextran sodium sulphate (DSS) models of colitis. These experiments are designed to test if delivery of TG-loaded colonic microbiota will ameliorate our mouse colitis models via phagocytosis or whether it is the altered (less dysbiotic) microbiota of TG-treated mice that ameliorates colitis in either Hprt-host deficient or WT animals. The experiments should discern if there is a therapeutic contribution from the TG-treated microbiota. Alternatively, a null result where all conventional mice have equally progressive colitis with TG-loaded bacteria and loaded bacteria controls, or germ-free Winnie evolve colitis whether gavaged TG-altered or unaltered microbiota, would suggest that there must be an alternative mechanism of action for TG to ameliorate colitis in Hprt-ko models.

**Non-canonical conversion of TG to TGTP**

The other logical explanation for the beneficial effect of TG in the absence of host Hprt is that there may be alternative hitherto undiscovered host enzyme pathways to salvage guanine base to GTP. This explanation was not explored in the Gut paper because alternative salvage pathway enzymes are not reported in the eukaryote literature. A search of the KEGG pathway database revealed that a guanosine kinase (gsk: EC: 2.7.1.73) is reported in the microbiology literature. Guanosine kinase (gsk) is a bidirectional ATP-dependent enzyme converting ATP  C guanosine  C ADP  C GMP. However, blasting revealed no gsk equivalent in the human and mouse genome and proteome NCBI databases.

In our 2013 Gut paper we demonstrated a statistically significant immunosuppression with 10 days of gavage of 2.5 mg/kg TG compared to vehicle gavage in Hprt-ko mice. The degree of immunosuppression was significantly less than in WT mice gavaged > 0.5 mg/kg/d TG, and we believe could have arisen from macrophage engulfment of gut bacteria carrying thioguanine nucleotides. We therefore asked whether daily intraperitoneal (i.p) TG as opposed to oral TG delivery over 7 days would have a reduced impact on WBC in wild-type mice due to the bypass of microbial luminal catabolism, but no significant difference was observed most likely due to catabolism by peritoneal macrophages. (Fig. 3). In the same pilot experiment with Hprt-ko mice, no statistical difference was detected between oral and i.p routes of drug delivery. The possible explanations for these results include that the microbiota do not contribute significantly to the immunosuppression, type II errors, absence of host salvage of TG in Hprt-ko, less bioavailability by the oral route, and catabolism by peritoneal macrophages. Given the inconclusive nature of these experiments, it remains a possibility that murine host cells could express alternative non-canonical purine salvage pathway enzymes to make thioguanine nucleotides even though no such pathway has been reported. However, if such a pathway existed then it could explain TG’s effect to ameliorate colitis in the absence of the universally expressed HPRT. We will test the null hypothesis that there is no alternative purine salvage pathway in the mammalian host that converts TG to TGN using Hprt-deficient germ-free mice and controls, and human cells deficient in HPRT.

The experiments will confirm if TG-loaded bacteria play a role in colonic TG treatment. Alternatively, or as well, demonstration of alternative guanine salvage will be of much interest to the wider scientific community where it has long been accepted that there is only one highly conserved purine salvage pathway in eukaryotes.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.
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