In vivo modification of tRNA with an artificial nucleobase leads to full disease remission in an animal model of multiple sclerosis

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ABSTRACT

Queuine is a modified pyrrolopyrimidine nucleobase derived exclusively from bacteria. It posttranscriptionally replaces guanine 34 in transfer RNA isoacceptors for Asp, Asn, His and Tyr, in almost all eukaryotic organisms, through the activity of the ancient tRNA guanine transglycosylase (TGT) enzyme. tRNA hypomodification with queuine is a characteristic of rapidly-proliferating, non-differentiated cells. Autoimmune diseases, including multiple sclerosis, are characterised by the rapid expansion of T cells directed to self-antigens. Here, we demonstrate the potential medicinal relevance of targeting the modification of tRNA in the treatment of a chronic multiple sclerosis model—murine experimental autoimmune encephalomyelitis. Administration of a *de novo* designed eukaryotic TGT substrate (NPPDAG) led to an unprecedented complete reversal of clinical symptoms and a dramatic reduction of markers associated with immune hyperactivation and neuronal damage after five daily doses. TGT is essential for the therapeutic effect, since animals deficient in TGT activity were refractory to therapy. The data suggest that exploitation of the eukaryotic TGT enzyme is a promising approach for the treatment of multiple sclerosis.

INTRODUCTION

Multiple Sclerosis (MS) is a debilitating inflammatory disease of the brain and spinal cord characterised by (*inter alia*) a rapid expansion of autoreactive T-cells that infiltrate into the CNS, resulting in focal plaques of demyelination and axonal injury (1). MS affects 2.3 million people worldwide, as either a relapsing-remitting or progressive form (2). Therapies that can fully prevent disease relapse and which can effectively control the disease course remain elusive (3).

Queuine is a pyrrolopyrimidine molecule that is exclusively synthesised by bacteria, but which is salvaged by eukaryotes—including humans—and incorporated into the wobble position (nucleoside 34) of transfer RNA (tRNA) containing a $G_{34}U_{35}N_{36}$ anticodon sequence; tRNA specific for the amino acids asparagine, aspartic acid, histidine and tyrosine (4,5). Queuine modification of tRNA is an irreversible event (6) and occurs through a unique base-for-base exchange reaction (guanine replacement by queuine) by the eukaryotic tRNA guanine transglycosylase (TGT) enzyme; also referred to as Queuine tRNA–ribosyltransferase (Figure 1A). Queuine deficiency has been variously associated with effects on proliferation, metabolism and tyrosine production (4,5). However, mice lacking TGT activity (*Qtrt1*$^{Gt/Gt}$ animals) are viable and largely asymptomatic (7).

Over three decades ago, the chemotherapeutic agent 6-thioguanine (6TG) was shown to act as a eukaryotic TGT substrate (6,8). 6TG itself is a powerful antiproliferative, cytotoxic agent whose principle mode-of-action is through its incorporation into DNA (9). The first and rate-limiting step in 6TG nucleotide conversion is catalysed by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Figure 1B). Significantly, studies on HPRT-deficient HL-60 myeloid cells showed that 6TG can reduce proliferation and induce differentiation (10); an effect that was later postulated to arise from tRNA incorporation by the TGT enzyme (11–14). Further unrelated studies suggest this secondary antiproliferative mechanism is likely to be responsible for only low systemic toxicity, since mice possessing a spontaneous mutation in the *Hprt* gene exhibited a lethal dose for 6TG that is 23-fold higher than that of wild-type animals (15).
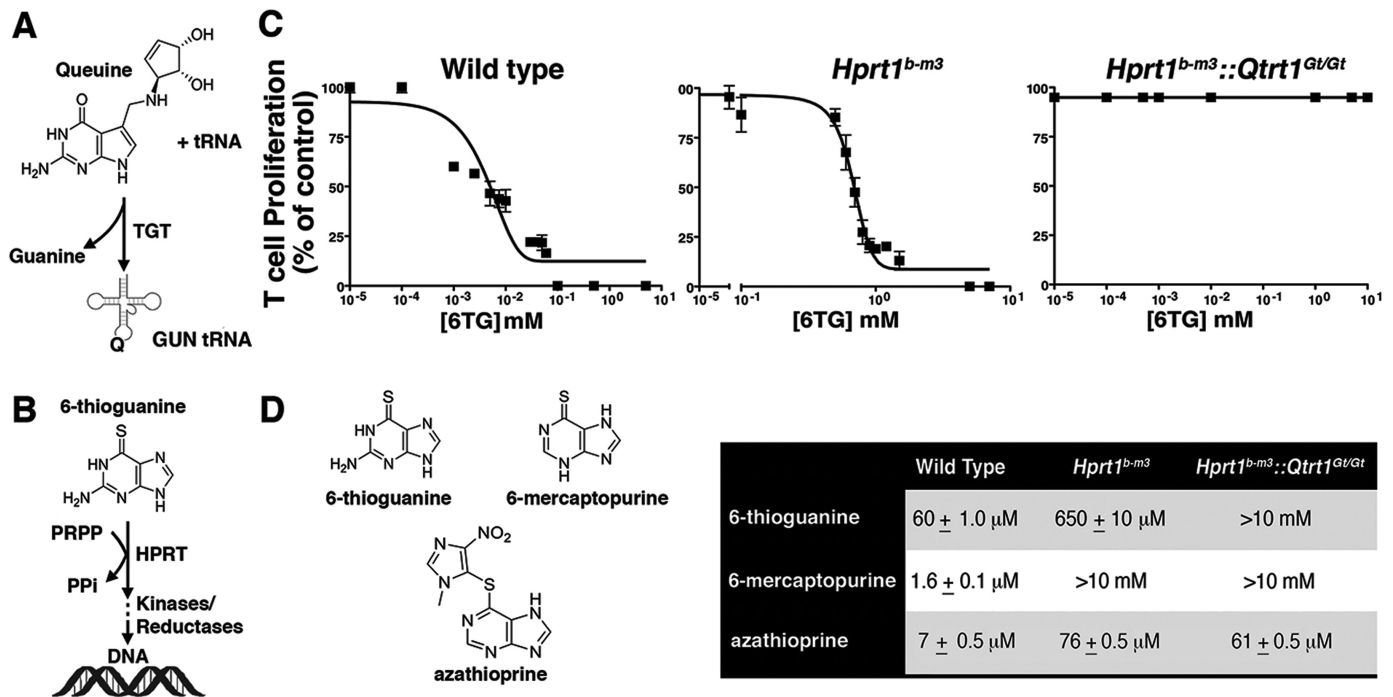
Figure 1. 6TG limits T cell proliferation *ex vivo* through the TGT pathway. (**A**) The TGT enzyme irreversibly replaces guanine with queuine base in the wobble position of tRNA containing a $G_{34}U_{35}N_{36}$ anticodon (where N is any nucleotide). (**B**) Hypoxanthine-guanine phosphoribosyltransferase (HPRT) is the first enzyme in the pathway that converts 6TG to Thio-GTP, prior to its incorporation into replicating DNA (Supplementary Figure S2). (**C**) CFSE-stained spleen cells from the indicated genotype of mouse were stimulated (anti-CD3ϵ and IL2) in the presence of titrating concentrations of 6TG. $CD3^+$ T cell proliferation was evaluated by flow cytometry using CFSE dye dilution and plotted as a percentage of non-treated controls. (**D**) EC_{50} values for thiopurine-mediated anti-proliferation of $CD3^+$ T cells in spleen cell cultures from the indicated genotype of animal. Mean ($\pm$ s.d.) of triplicates. Representative of three independent experiments. PRPP, phosphoribosyl pyrophosphate; PPi, pyrophosphate.

It has been observed that the tRNA of differentiated cells in adult tissue are substantially—and irreversibly—modified by queuine, whereas the tRNA of regenerating liver (and foetal liver) and that of rapidly proliferating cells, including numerous tumour types, are under-modified and therefore susceptible to base modification *via* TGT (5). Therefore, we investigated whether the modification of tRNA with an artifical nucleobase could limit the activity of rapidly proliferating immune cells. Here, we show that forced incorporation of either 6TG or a *de novo* designed nucleobase into tRNA leads to a striking reversal of clinical symptoms and neuronal inflammation in the murine MS model experimental autoimmune encephalomyelitis (EAE). The therapeutic effect arises from a dramatic reduction in effector immune cell proliferation and activation, without impacting the resting and post-proliferative cell populations. Our findings suggest that strategies that target the eukaryotic TGT enzyme may have the potential to treat multiple sclerosis and other T cell mediated autoimmune diseases.

MATERIALS AND METHODS

Animals

C57BL/6 (Harlan, UK), B6.129P2-*Hprt1*$^{b-m3}$/J (Jackson Laboratories), B6.129S4-*Qtrt1*$^{Gt/Gt}$ (7) and B6;129-*Hprt1*$^{b-m3}$:: *Qtrt1*$^{Gt/Gt}$ (N5F3) mice were bred in specific pathogen-free conditions and experiments were conducted under license from the Irish Department of Health and Children (Ref: B100/3801) and with the approval of Trinity College Dublin Animal Research Ethics Committee (Ref: 241109 and 111012).

Cell culture and EC_{50} determination

Single cell splenocyte suspensions were stained with 1 μM CFSE (Molecular Probes), quenched with 10% horse serum and washed with PBS. Cell pellets were resuspended in XVivo™ 15 medium (LONZA) and plated at 1×10^6 cells/ml in 24 well plates coated with 2 μg/ml anti-CD3ϵ (BD Biosciences). Cells were stimulated with 5 ng/ml human recombinant IL-2 (RnD systems) every 48 h starting on day 0. On day 5, cells were stained with Pe-Cy7 CD3ϵ antibody (BD Pharmingen) for 20 min followed by 1 μg/ml Propidium Iodide (Sigma) prior to analysis by flow cytometry (Canto, BD Biosciences). Data were analysed using FlowJo software. Queuine (a gift from Dr Susumu Nishimura, Japan), guanine, 6TG, 6-mercaptopurine and azathioprine were added to cultures day 1 post-plating to examine antiproliferative activity.

EAE induction, compound administration and disease evaluation

Chronic, monophasic EAE was induced in 8- to 10-week old female mice by sub-cutaneous (s.c.) injection of 200 μl emulsion containing 150 μg MOG$_{33-55}$ peptide (Genscript) in Complete Freund's Adjuvant (containing 5 mg/ml

heat-inactivated *Mycobacterium tuberculosis*). On the same day, mice were administered 500 ng Pertussis Toxin (Kaketsuken, Japan) intraperitoneally (i.p.) and again two days later. 6TG (*Lanvis®*) was made to a concentration of 2.66 mg/ml in 7.5 mM NaOH in phosphate buffered saline (PBS) and filter sterilised prior to injection (15 mg/Kg; 100 μl volume). NPPDAG was dissolved in DMSO and administered to animals as a diluted, filter sterilised solution in PBS (4% DMSO final; 30 mg/Kg, 200 μL volume). Queuine stock was dissolved in H_2O and administered to animals as a diluted, filter sterilised solution in PBS (30 mg/Kg, 100 μl volume). Animals were administered terminal anaesthetic (40 μl of euthanol) and perfused intracardially prior to tissue harvest. Disease severity was recorded every 24 h: 0-Normal; 1-flaccid tail; 2-imparied/wobbly gait; 3-complete hind limb weakness; 4-hind limb and forelimb paralysis; 5-moribund state/dead. Weight loss and the ability to suspend from a horizontal bar were assessed at indicated intervals as described previously (16).

Immunohistochemistry

Whole brain from heparin-PBS perfused mice were embedded in OCT freezing medium over liquid nitrogen. The brain stem and cerebellum were sectioned by cryostat (10 μm thickness) onto slides coated with APS (3-Aminopropyltriethoxy-silane) and stained for FA11-CD68 and KT3-CD3 using the avidin–biotin–peroxidase complex method (ABC, Thermo Scientific); 0.015% (*v/v*) hydrogen peroxide as substrate and diaminobenzidine (Sigma) as chromagen and counterstained with haemotoxylin (Sigma). Slides were dewaxed in Histoclear I and II (ABC Scientific) and coverslips were mounted with DPX mountant (Sigma). All pictures were taken using Cell-1 software (Leica).

Analysis of mononuclear cells (MNC) from brain

Whole brain from ice-cold PBS perfused mice were isolated, filtered through a 70 μm pore size nylon mesh and digested with 1 mg/ml collagenase D and 10 μg/ml DNase I for 1 h at 37°C. The single cell suspension was washed and applied to a five-layer Percoll (GE healthcare) gradient and centrifuged at $1250 \times g$ for 45 min at 18°C. Mononuclear cells (MNCs) were isolated at the interface between 1.088:1.072 g/ml and 1.072:1.030 g/ml.

Antibodies and FACS analysis

For cell surface staining, MNCs and splenocyte suspension cells were labelled with the following antibodies (BD Phamingen and eBioscience): anti-CD3-FITC, anti-CD4-PeCy7, anti-CD8-PE C7, anti-F4/80-PeCy5, anti-Ly-6G-PeCy7, anti-δγ-TCR-APC, anti-CD11b-PE, anti-CD62L-APC, anti-CD44-PE, anti-CD19-AlexaFluor450, anti-CD8a-PerCP-Cy5.5 and anti-CD4-AlexaFluor780. All staining was carried out in the presence of Fcγ Block (Clone 93). For intracellular staining, cells were stimulated with PMA (10 ng/ml), ionomycin (1 μg/ml) and Brefeldin A (5 μg/ml) for 6 h at 37°C. Cells were stained for surface markers, then fixed and permeabilized using the Fix and Perm Cell Permeabilization Kit (Thermo Fisher Scientific).

Cells were labelled with anti-IL-17a-PE and anti-IFNγ-PerCP-Cy5.5 according to the manufacturer's instructions. Data were collected on a FACS Canto machine (BD Biosciences) and analysed using FlowJo software (Stanford University).

Antigen recall assay

Single cell splenocyte suspensions were seeded at 1×10^6 per ml into U-bottomed 96 well plates for 72 h containing either medium alone (negative control), or 10 ng/ml PMA and 1 μg/ml anti-CD3ε (positive control), or 2, 10 or 50 μg/ml MOG. Cells were pulsed with 0.5 μCi [methyl-^{3}H]-thymidine per well (Amersham) in RPMI containing 10% fetal bovine serum (FBS) for 16 h at 37°C. Cells were harvested onto glass fiber mats using an automated cell harvester and read on the micro-β-counter (Wallac) with 5 ml BetaScint (Wallac). Samples were assayed in triplicate and the results expressed as mean counts per minute (cpm).

Spinal cord transcript analysis

Spinal cords were extracted from heparin-PBS perfused mice. In accordance with manufacturers instructions, RNA was isolated using the RNeasy Plus Universal Tissue Mini Kit (Qiagen), reverse transcribed *via* the RT^2 First Strand Kit (Qiagen) and qPCR performed with a Custom RT^2 Profiler PCR Array (SabBiosciences; Qiagen) on an ABI 7500 Fast real-time PCR system (Applied Biosystems). Data were graphed on the RT^2 Profiler PCR Array Data Analysis Webportal with fold-changes in expression normalised against glucose 6-phosphate dehydrogenase (gGpdh).

tRNA [^{14}C] guanine displacement assay

To identify substrates for tRNA incorporation, the displacement of [^{14}C] guanine from pre-charged tRNA by the human TGT enzyme was evaluated in the presence of test compound. Human TGT was produced by co-expression of an *N*-terminal polyhistidine tagged QTRT1 and a *C*-terminal SUMO-StrepII tagged QTRT2 in BL21(DE3) *tgt*::Km$_r$ cells.

tRNA from Baker's yeast (Roche) was pre-labelled with [8-^{14}C] guanine-HCl (Moravek Biochemicals; 46.4 mCi/mmol stock) using an *N*-terminal tagged *E. coli* tRNA guanine transglycosylase (TGT) enzyme; described previously (17). A 10 μg quantity of *E. coli* TGT was added to a 150 μl tRNA labelling mix comprising, 50 mM Tris-HCl pH 7.5, 20 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 25 absorbance units (260 nm) of yeast tRNA, 144 μM [^{14}C] guanine. The reaction was incubated for 2 h at 37°C, made up to 400 μl with 50 mM Tris-HCl pH 7.5 and tRNA extracted by the addition of an equal volume (400 μl) of acid phenol:chloroform (5:1, pH 4.5; Ambion). Organic and aqueous phases were separated by centrifugation at 16 000 × *g* for 5 min. The radiolabelled tRNA with [8-^{14}C] guanine in the third position of the anticodon loop (tRNA*) was precipitated from the aqueous phase by the addition of 0.1 volume (40 μl) of 3 M sodium acetate (aq.) and 2 volumes of ethanol (800 μl) and incubated overnight at −20°C. The tRNA* was pelleted by centrifugation at 16 000 × *g* for

20 min at 4°C, washed with 1 ml of ice-cold 70% ethanol and redissolved in 20 μl nuclease-free water.

tRNA-[14C] guanine displacement assays contained 50 mM Tris-HCl, pH 7.5, 20 mM NaCl, 5 mM MgCl₂, 2 mM dithiothreitol, 2 μg human TGT enzyme, to which 200 μM of guanine, queuine, NPPDAG or 7 methylguanine were added. Reactions were initiated by the addition of 2 absorbance units (260 nm) of tRNA* and incubated for 1 h at 37°C.

Separation of tRNA* from free nucleobases was achieved by centrifugation of the reaction (0.1 × g) through a 1 ml final bed volume of Whatmann DEAE 52-cellulose resin (pre-equilibrated with 200 mM Tris-HCl pH 7.5) in 1.5 ml spin columns. The flow-through was collected and reloaded five times. Unbound material was washed from the column with 4 ml of Wash Buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 200 mM NaCl). Wash steps were pooled and collected into scintillation vials. The bound tRNA was eluted with 1 ml Elution Buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 M NaCl) and also collected into scintillation vials. A 10 ml volume of scintillation cocktail (Ecoscint A) was added to wash and elution fractions and counted for displaced [14C] guanine. Maximum displacement by 200 μM queuine, the natural eukaryotic TGT substrate, was 240 pmol [14C] guanine. Background values were ≤10 pmol.

tRNA-[14C] guanine incorporation assay

The ability of human TGT to insert [14C] guanine into the anticodon loop of tRNA that had been modified with guanine, queuine or NPPDAG was used to assess the reversibility of the modification. In an identical manner to the conditions described above for the tRNA-[14C] guanine displacement assay, tRNA was modified with 200 μM each of the non-labelled nucleobase (guanine, queuine or NPPDAG) using 10 μg of human TGT, phenol extracted and redissolved in nuclease-free water. Each incorporation assay contained 2 absorbance units (260 nm) of guanine-, queuine- or NPPDAG-modified tRNA and 2 μM [14C] guanine. Following a 1 h incubation the tRNA and nucleobases were separated on DEAE cellulose, the tRNA eluted and [14C] guanine-modified tRNA analysed by liquid scintillation counting.

Cellular incorporation assays for queuine and NPPDAG

In the case of human MDA-MB-231 cultures, cells were grown in serum free (queuine deficient) medium (5) in a 12-well dish containing 0, 50, 250, 500 nM queuine or NPPDAG for 72 h, washed with fresh medium and 25 nM [3H] queuine (Moravek Biochemicals; 8.9 Ci/mmol) added for a further 24 h. For immune cell analysis, single cell splenocyte suspensions were generated from EAE-disease mice (clinical score of 2) and plated onto 10 cm dishes (3 × 10⁷ cells per dish) in X-vivo™ 15 medium, followed by the addition of MOG antigen to a final concentration of 50 μg/ml. After 48 h, 200 μM of queuine or NPPDAG was added and at 72 h, cells were washed with fresh medium and 100 nM [3H] queuine added.

In both cases, cells were harvested at 96 h, lysed by the addition of 125 μl ice-cold 30 % TCA and incubated on ice

for 10 min. Precipitates were collected by vacuum filtration onto 2.4 cm GFA glass fibre disks, rinsed with ice-cold 5% TCA, then ice-cold 95% ethanol, dried in air and placed in scintillation cocktail (Ecoscint A) for counting.

HPRT activity assay

HPRT reaction mix (20 mM HEPES, pH 8.0, 10 mM MgCl₂, 0.1 mM phosphoribosyl pyrophosphate, 20 μM [8-14C]-guanine made to a volume of 100 μl was prepared in the absence or presence of 400 μM queuine, guanine or NPPDAG and preheated to 37°C before addition of 10 ng recombinant human HPRT enzyme. Reactions were terminated after 1 h by rapid heating to 100°C. Control reactions comprised 1 M HCl-inactivated enzyme neutralised by 1 M NaOH prior to addition to the reaction. After inactivation, reactions were cooled to room temperature and applied to a 1 ml packed resin of Dowex-1×8 (pre-charged with 1 M HCl and equilibrated with H₂O). Columns were washed with 5 ml milli-Q H₂O and 10 ml 10 mM HCl. GMP was eluted with 5 ml of 50 mM HCl into 15 ml of scintillation cocktail (Ecoscint A) and analysed by scintillation counting.

Synthesis of *N*-((2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)methyl)-3-phenylpropan-1-aminium chloride (NPPDAG)
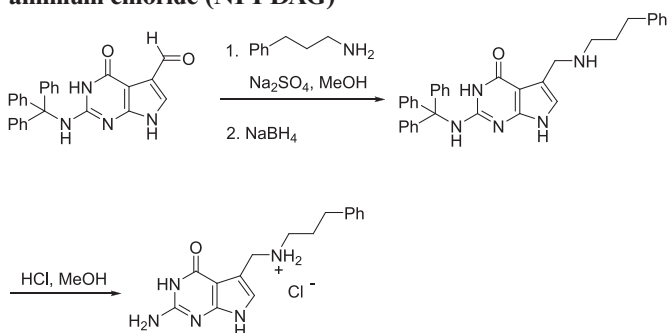
Preparation of 5-((3-phenylpropylamino)methyl)-2-(tritylamino)-3*H*-pyrrolo[2,3-*d*]pyrimidin-4(7*H*)-one
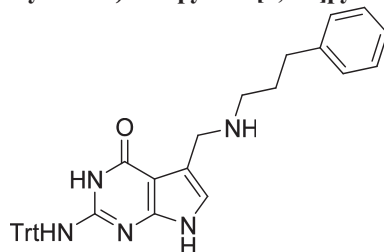
To a suspension of 4-oxo-2-(tritylamino)-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-5-carbaldehyde (200 mg, 0.48 mmol) and sodium sulfate (5 mg) in methanol (5 ml) under an argon atmosphere was added 3-phenylpropylamine (74 μl, 0.52 mmol) and the resulting suspension stirred at room temperature for 2 h. Sodium borohydride (55 mg, 1.5 mmol) was then added and the reaction mixture stirred at room temperature for a further 1 h. Water (5 ml) was added and the resulting suspension stirred for 10 min before being extracted with dichloromethane (3 × 5 ml). The combined organic layers were dried (MgSO₄) and concentrated *in vacuo* to yield the crude product that was purified by flash chromatography (9:1 dichloromethane-MeOH) to yield the

desired compound as a white solid (210 mg, 81%), m.p. > 300°C (decomposition).

^{1}H (400 MHz, DMSO-d$_6$): δ 1.73 (apparent quintet, J = 7.8 Hz, 2H), 2.56 (m, 4H), 3.74 (s, 1H), 6.42 (s, 1H), 7.19 (m, 20H), 7.45 (bs, 1H), 10.78 (bs, 1H).

^{13}C (400 MHz, DMSO-d$_6$): δ 31.7, 32.8, 45.4, 47.8, 70.4 (q), 99.7 (q), 114.9, 117.6 (q), 125.9, 126.0, 126.9, 128.0, 128.6, 129.0, 142.6 (q), 145.4 (q), 150.0 (q), 150.4 (q), 159.7 (C = O).

HRMS (ESI$^+$) m/z: Found: 540.2757 [M+H]$^+$ C$_{35}$H$_{34}$N$_5$O Requires: 540.2765.

ν_{max}/cm^{-1}: 1542, 1611, 1670, 2868, 2951.

Preparation of *N*-((2-amino-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)methyl)-3-phenylpropan-1-aminium chloride (NPPDAG)
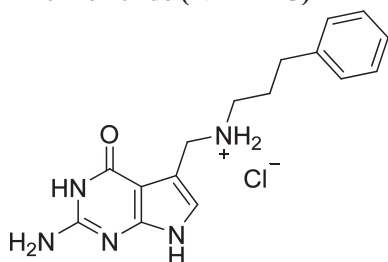
To 5-((3-phenylpropylamino)methyl)-2-(tritylamino)-3*H*-pyrrolo[2,3-*d*]pyrimidin-4(7*H*)-one (210 mg, 0.39 mmol) was added methanolic HCl (1.25 M, 3 ml). The resulting solution was stirred at room temperature for 16 h. The precipitated product was removed by vacuum filtration and washed with dichloromethane to yield the desired compound as a white powder (84 mg, 65%), m.p. > 300°C (decomposition).

^{1}H (600 MHz, DMSO-d$_6$): δ 1.89 (apparent quintet, 2H), 2.61 (t, J = 7.8 Hz, 2H), 2.82–2.93 (m, 2H), 4.08–4.15 (m, 2H), 6.50 (bs, 2H), 6.82 (s, 1H), 7.12–7.19 (m, 3H), 7.22–7.28 (m, 2H), 9.15 (bs, 2H), 11.19 (m, 1H), 11.39 (bd, 1H).

^{13}C (150 MHz, DMSO-d$_6$): δ27.7, 32.3, 43.0, 45.6, 98.7, 108.8, 117.9,126.5, 128.7, 128.9, 141.1, 152.6, 153.4, 160.7.

HRMS (ESI$^+$) m/z : Found: 298.1662 [M]$^+$ C$_{16}$H$_{20}$N$_5$O Requires: 298.1664.

IR(film) ν_{max} /cm^{-1}: 1456, 1625, 2443, 2713, 2756, 2873, 2933, 3184.

RESULTS

Insertion of 6-thioguanine into tRNA leads to full remission in EAE

In order to isolate the tRNA-related anti-proliferative effects of 6TG on immune effector cells, T cell proliferation was examined in spleen cell cultures from wild-type, HPRT deficient (*Hprt1^{b-m3}*) and double knockout *Hprt1^{b-m3}::Qtrt1$^{Gt/Gt}$* mice (Figure 1C). Treatment with 6TG led to a dose-dependent inhibition of T cell proliferation in wild-type (EC$_{50}$ 60 μM) and *Hprt1^{b-m3}* cells (EC$_{50}$ 650 μM) whereas T cells from *Hprt1^{b-m3}::Qtrt1$^{Gt/Gt}$* mice, lacking both TGT and HPRT activity, were completely refractory to 6TG anti-proliferative effects up to 10 mM. The data demonstrate that 6TG can mediate a potent antiproliferative effect on T cells through a HPRT independent mechanism and which is reliant on the TGT enzyme.

In contrast to 6TG, neither 6-mercaptopurine nor azathioprine (antiproliferative agents of the thiopurine family) possess secondary drug activity through the TGT enzyme (Figure 1D and Supplementary Figure S1A and B) and fail to be incorporated into tRNA (Supplementary Figure S1C); instead relying mechanistically on nucleotide conversion by HPRT to exert their antiproliferative effects (Supplementary Figure S2). As expected, guanine had no influence on the proliferation of T cells from mice of all three genotypes (Supplementary Figure S3).

Expanding the findings to an *in vivo* setting, EAE was induced by immunising *Hprt1^{b-m3}* mice with myelin oligodendrocyte glycoprotein (MOG$_{33-55}$) peptide in complete Freund's adjuvant to induce a murine model of chronic MS. Upon reaching a clinical score of two—characterised by a limp tail and wobbly gait and typically 9 days post immunisation (dpi)—the administration of 6TG i.p., dramatically arrested and reversed the disease to a state visually indistinguishable from control animals by 21 dpi (Figure 2A and Movie S1). Quantitative and observer-independent proof of the therapeutic effect was provided by data showing a rate of weight gain and levels of motor co-ordination (horizontal bar test) similar to non-diseased controls (Figure 2B and C). The administration of 6TG did not affect the course of EAE in *Hprt1^{b-m3}::Qtrt1$^{Gt/Gt}$* double knockout mice, as these animals presented with identical clinical disease scores, weight loss and performance in the horizontal bar test as non-treated mice with EAE (Figure 2D–F). Together, the data verify that the HPRT-independent therapeutic effect of 6TG relies exclusively on the TGT pathway.

6TG modified tRNA selectively inhibits effector immune cell function in EAE

To shed some light on the processes associated with the remarkable recovery of treated animals, the infiltration of the CNS by T cells and the degree of activation of resident microglia, which are key characteristics of EAE and MS ([18],[19]), were examined (Figure 2G). Immunohistochemical analysis of the CNS of both naïve and EAE induced, 6TG-treated *Hprt1^{b-m3}* mice using the late endosomal marker CD68 showed an absence of activated microglia and activated macrophages whereas staining for the pan-T cell marker CD3 resulted in no detectable infiltration by T cells (rightmost panels). Untreated *Hprt1^{b-m3}* mice with EAE exhibited the expected degenerative immunohistochemical profiles associated with immune cell infiltration (centre-right panels).

In EAE and MS, neuronal degeneration is initiated by primed immune cells that originate from the periphery ([1],[20]). Therefore, the effect of 6TG treatment on lymphoid and myeloid populations—including the MS-relevant T$_H$1 and T$_H$17 T cell populations ([18])—in the brain and the periphery were investigated. Intracellular cytokine staining demonstrated a systemic decrease in pathogenic effector T$_H$1, T$_H$17 (Figure 2H), γδ and CD8$^+$ cytotoxic T cells (Supplementary Figure S4A) in both the brain and spleen of treated *Qtrt1$^{Gt/Gt}$* mice. Interestingly, B cells were affected in a similar fashion in the spleen, but not the brain. Importantly, treatment with 6TG alone did not induce general, unselective lymphocyte depletion (lympho-
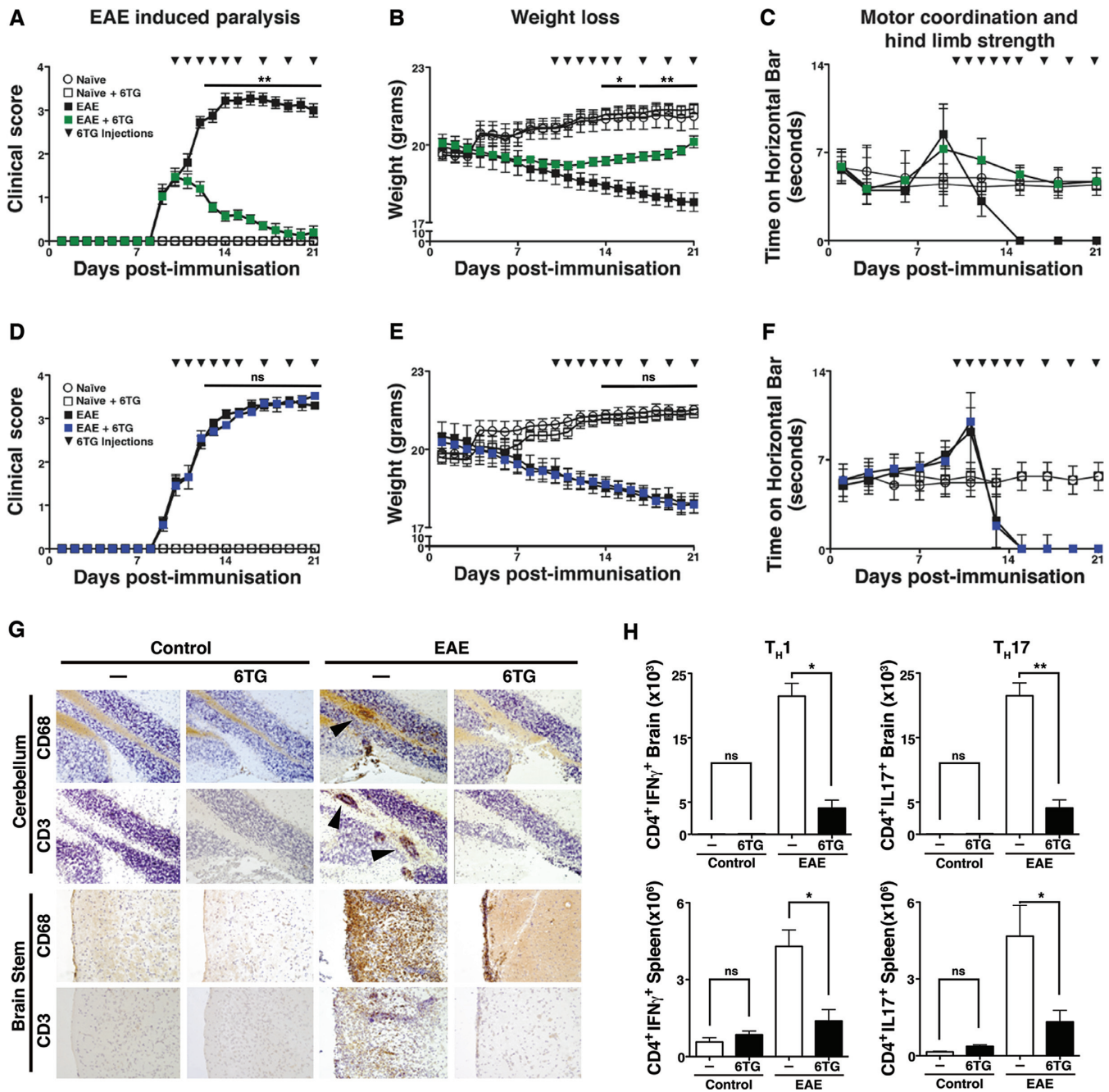
Figure 2. 6TG administration leads to EAE disease remission in *Hprt1*$^{b-m3}$ mice. (**A–C**) Control non-diseased (Naïve) and EAE induced (EAE) *Hprt1*$^{b-m3}$ and (**D–F**) *Hprt1*$^{b-m3}$*::Qtrt1*$^{Gt/Gt}$ female mice were administered PBS or 6TG (15 mg/Kg) i.p. in accordance with the treatment regime shown by the inverted arrowheads. (**A** and **D**) Animals were monitored daily for clinical score. Data represent the mean ± s.e.m. $n = 8$ per group. Consistent across three separate experiments. *$P < 0.05$, **$P < 0.001$ by Mann–Whitney's U test for EAE clinical score in mice treated with 6TG (EAE + 6TG) compared to controls (EAE). (**B** and **E**) Daily weight of the animals. Repeated measures ANOVA with *post-hoc* pair-wise comparisons revealed that the weight of 6TG treated EAE-induced mice (EAE + 6TG) were significantly different from (**B**) EAE diseased mice (EAE) beginning 14 dpi, conversely, the weight of *Hprt1*$^{b-m3}$*::Qtrt1*$^{Gt/Gt}$ 6TG treated EAE-induced mice (EAE + 6TG) were not significantly different from EAE diseased mice (EAE), *$P < 0.05$, **$P < 0.001$. ns, not significant. (**C** and **F**) Animals were evaluated every third day for motor coordination on the horizontal bar test. (**G**) Sections of fresh frozen cerebellum and brain stem from perfused mice were stained for CD68 and CD3 and counter stained with haematoxylin. (**H**) Mononuclear cells isolated from perfused brain and spleen were re-stimulated *in vitro* (PMA, Ionomycin) in the presence of Brefeldin A and the populations of T$_H$1 (CD3$^+$CD4$^+$IFN$_\Upsilon$$^+$) and T$_H$17 (CD3$^+$CD4$^+$IL17$^+$) cells analysed by flow cytometry. Mean (± s.d.) for triplicate samples. $n = 4$ animals per group. *$P < 0.05$, **$P < 0.001$, t-test.
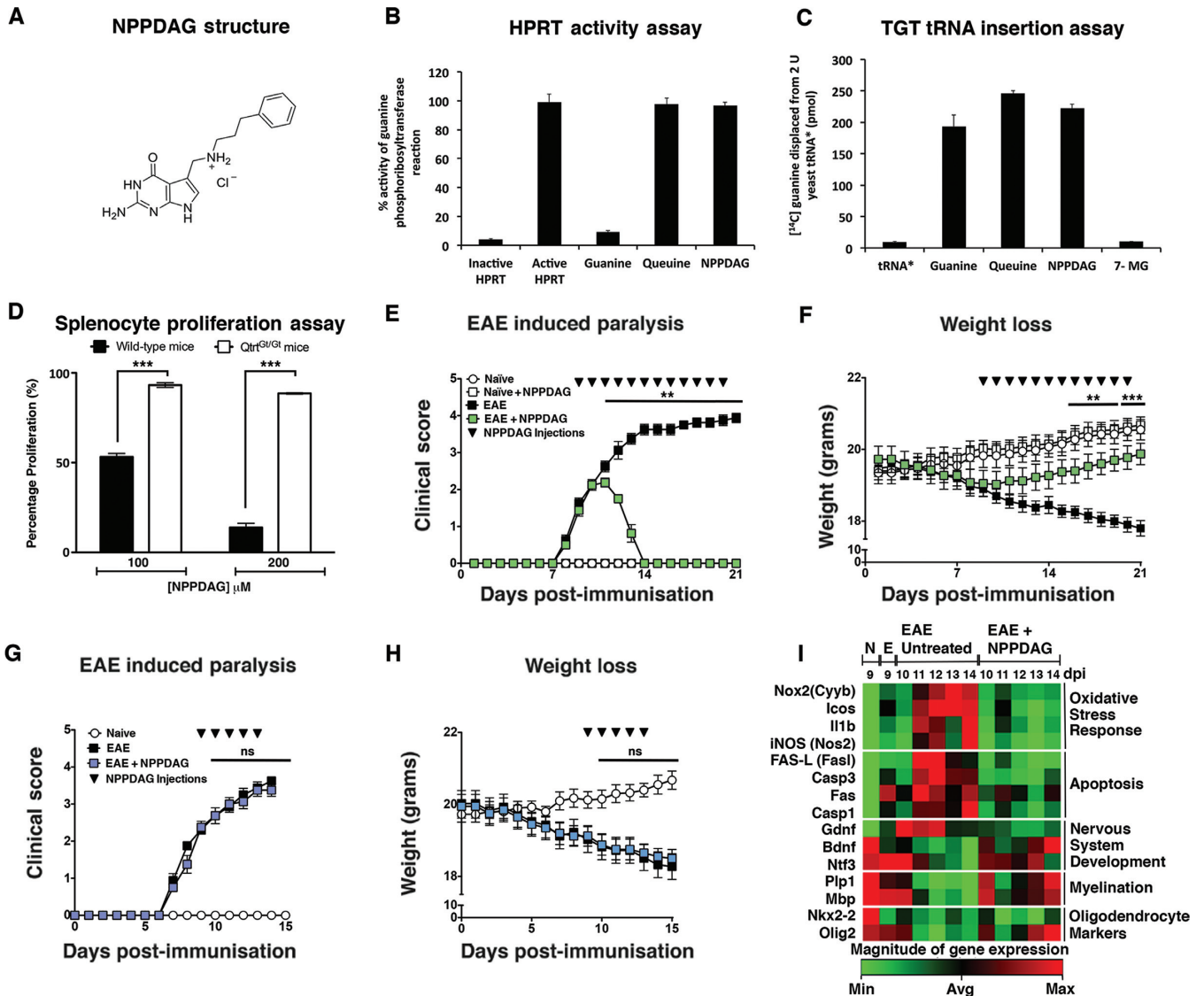
Figure 3. NPPDAG is a TGT-specific substrate that rapidly reverses disease in EAE. (**A**) Chemical structure of NPPDAG. (**B**) The production of GMP from radiolabelled guanine (20 μM) by recombinant human HPRT was examined in the absence or presence of a 20-fold excess of unlabeled guanine, queuine or NPPDAG (400 μM). Queuine and NPPDAG showed no effect on GMP production indicating they are neither substrates nor inhibitors. (**C**) Guanine, queuine and NPPDAG act as substrates for the eukaryotic TGT enzyme as indicated by their ability to displace radiolabelled guanine from position 34 of the anticodon loop of precharged tRNA (tRNA*), whereas 7-methylguanine (7-MG) does not act as a substrate. (**D**) Spleen cells from wild-type (black bars) and $Qtrt1^{Gt/Gt}$ mice (white-bars) were stained with CFSE, stimulated (anti-CD3ε and IL2) and treated with the indicated concentrations of NPPDAG for 72 h. CD3$^+$ T cell proliferation was evaluated by flow cytometry using CFSE dye dilution. Mean ($\pm$ s.d.) as percentage of control for triplicate samples. ***$P < 0.001$, *t*-test. (**E** and **F**) Control (Naïve) and EAE induced (EAE) wild-type or (**G** and **H**) $Qtrt1^{Gt/Gt}$ female mice were administered PBS or NPPDAG (30 mg/Kg) i.p. in accordance with the treatment regime shown by the inverted arrowheads. (**E** and **G**) Clinical symptoms were monitored daily. Data represent the mean $\pm$ s.e.m. $n = 8$ per group. Data consistent across three separate experiments. **$P < 0.05$ by Mann–Whitney's U test for EAE scores (clinical score) in mice treated with NPPDAG (EAE + NPPDAG) compared to controls (EAE). ns, not significant. (**F** and **H**) Daily weight of the animals. Repeated measures ANOVA with *post-hoc* pair-wise comparisons revealed that the weight of NPPDAG treated EAE-induced wild-type mice (**F**, EAE + NPPDAG) were significantly different from EAE diseased mice (EAE), **$P < 0.05$, ***$P < 0.001$. ns, not significant. (**I**) qRT-PCR analysis of transcript extracted from the spinal cord of perfused non-diseased (N), EAE induced (E) and EAE induced animals administered vehicle only (EAE untreated) or NPPDAG (EAE + NPPDAG). Each data point is the average of three independent animals for each day (10–14 dpi), normalised to gGpdh control.
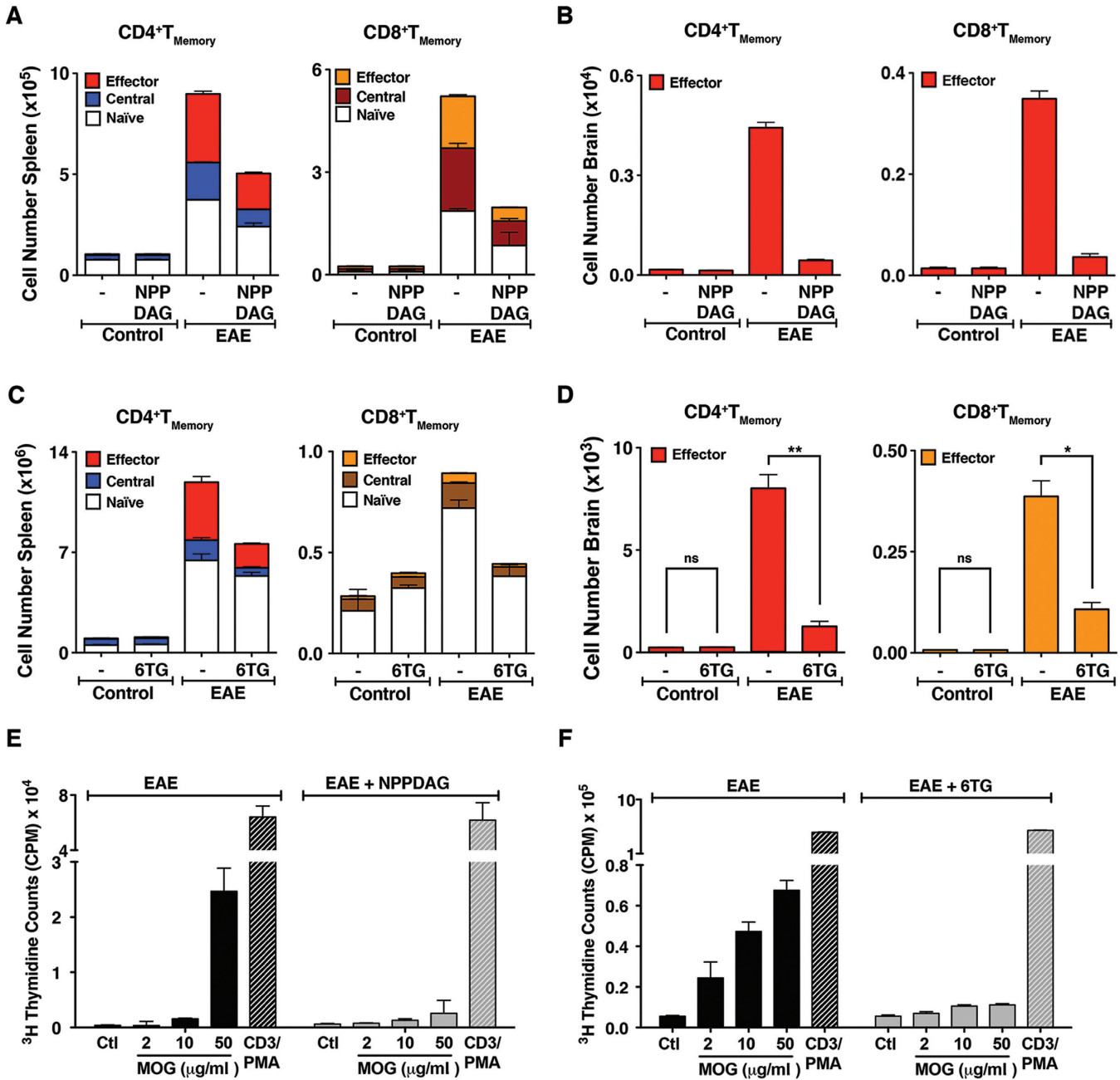
Figure 4. NPPDAG and 6TG limit antigen-specific effector responses in EAE. (**A** and **B**) Non-diseased (control) and EAE induced (EAE) wild-type and (**C** and **D**) *Hprt1[b-m3]* female mice were administered PBS (-), NPPDAG (30 mg/Kg) or 6TG (15 mg/Kg) upon reaching a clinical score of two, as indicated. In the NPPDAG and 6TG studies, unstimulated spleen cells and mononuclear brain cells were isolated from perfused animals after full remission, at 15 dpi and 21 dpi, respectively, and labeled for naïve (CD3+62L^{hi} CD44^{lo}), central memory (T_{CM}; CD3+62L^{hi} CD44^{hi}), and effector memory (T_{EM}; CD3+CD62L^{lo} CD44^{hi}) cell sub-populations in both the CD4+ and CD8+ cell pools. Mean (± s.d.) for triplicate samples. *n* = 4 animals per group. *$P <$ 0.05, **$P <$ 0.001, *t*-test. (**E**) Spleens from perfused wild-type and (**F**) *Hprt1[b-m3]* EAE induced animals that were untreated or treated with the indicated compounds were plated with increasing concentration of MOG$_{35-55}$ antigen at 2, 10 and 50 μg/ml (solid bars) or anti-CD3ε/PMA as mitogenic control (hatched bars). At 72 h cells were pulsed with [^{3}H] thymidine for 16 h to assay for antigen-dependent proliferation. Mean (± s.d.) counts per minute (cpm) for triplicate samples. *$P <$ 0.05, *$P <$ 0.001, ns, not significant, one-way ANOVA followed by Bonferroni test for differences in MOG$_{35-55}$ induced proliferation compared to control.

cytopenia). Instead, a marginal elevation in the number of immune cells within the periphery and brain of treated naïve mice was observed in some cases. As expected, in untreated, diseased animals there was a significant infiltration of T_H1 and T_H17 CD4$^+$, CD8$^+$ and γδ T cells into the brain and expanded cell numbers in the spleen. The induction of EAE, and MS in humans, strictly depends on the activation of an innate immune response that contributes to inflammatory cell recruitment, demyelination and the induction of pathogenic T_H1 and T_H17 cells (18,19). Therefore, the innate immune cell populations of mature macrophages/monocytes (F4/80), myeloid cells (CD11b) and neutrophils (Ly6G) were also examined (Supplementary Figure S4B). In all cases, EAE was associated with infiltration of the brain by these cell types, which was dramatically limited by 6TG treatment.

The *de novo* designed nucleobase NPPDAG rapidly induces full disease reversal in EAE

Attention next turned to the problem of the HPRT-mediated toxicity associated with 6TG, which precludes its use as an MS treatment. Surprisingly, administration of queuine to wild-type mice with chronic EAE caused an arrest in disease progression but failed to reverse symptoms over time (Supplementary Figure S5). Since queuine is not a substrate for HPRT (21), a search for a competent TGT substrate not recognised by HPRT was initiated, centering on the 7-deazaguanine core. This led to the identification of NPPDAG (Figure 3A and Supplementary Figure S6), which was inert as a substrate for HPRT (Figure 3B) but readily incorporated into tRNA *in vitro* in an irreversible manner by the TGT enzyme (Figure 3C and Supplementary Figure S7A). Both human MDA-MB-231 cells and spleen cells isolated from EAE-diseased animals were found to incorporate NPPDAG into RNA, which strongly suppressed subsequent modification by queuine; confirming that the intracellular incorporation of NPPDAG into RNA is likewise irreversible. NPPDAG exhibited a significant anti-proliferative effect on T cells that was dependent on the TGT enzyme (Figure 3D). *In vivo*, treatment of EAE diseased mice with NPPDAG—upon reaching a clinical score of two—dramatically arrested and reversed the disease course after only five daily doses (30 mg/Kg) to a state indistinguishable from non-diseased controls (Figure 3E and Movie S2). Again, mice resumed a normal rate of weight gain (Figure 3F) and ability to cross a horizontal bar similar to control, non-diseased animals (Supplementary Figure S8). Consistent with the earlier *in vitro* data, NPPDAG had no effect on disease progression in *Qtrt1$^{Gt/Gt}$* mice (Figure 3G and H).

A number of neuronal repair pathways are known to control inflammation and tissue damage in EAE and MS (18,22–23). In order to provide confirmation of the apparent phenotypic recovery of the treated animals, an analysis of spinal cord samples by quantitative reverse transcriptase PCR (qRT-PCR)-array was performed (Figure 3I and Supplementary Figure S9). EAE was associated with a robust transcriptional up-regulation of selected genes associated with oxidative stress (iNOS, Nox2) and apoptosis (Casp1, Casp3, FasL), and a down regulation of markers associated

with neuronal development (BDNF, NT-3) and myelination (Plp1, Mbp) relative to non-diseased controls. Treatment of diseased mice with NPPDAG was associated with a return to normal levels of expression of these markers.

In EAE and MS, persistent immunological activity is exacerbated by antigen-specific long-lived effector memory (T_{EM}) and central memory (T_{CM}) T cell subsets (24–29). The frequency of T_{EM} and T_{CM} cells in the spleen was significantly reduced by NPPDAG in EAE induced wild-type mice (Figure 4A). A striking decrease in the number of T_{EM} cells in the brain was also observed (Figure 4B). Importantly, the expanded peripheral populations of naïve CD4$^+$ or CD8$^+$ T cells in mice with EAE were not depressed below levels observed in normal, non-diseased animals. Administration of 6TG to disease-induced *Hprt1^{b-m3}* animals mirrored the therapeutic effect of NPPDAG (Figure 4C and D).

Importantly, the effect of both compounds is not of a general immunosuppressive nature. In both cases, spleen cells proliferated robustly in response to polyclonal activation with anti-CD3ε and phorbol 12-myristate 13-acetate across the non-treated and treated EAE induced mice (Figure 4E and F; hatched bars). Administration of increasing MOG$_{33-55}$ concentrations (2–50 μg/ml) led to a dose-dependent proliferation of spleen cells from mice with EAE, yet failed to induce the proliferation of spleen cells from mice treated with either NPPDAG or 6TG (black and grey bars). The experimental data show that both NPPDAG and 6TG act through the eukaryotic TGT pathway to systemically limit the number and activity of encephalitogenic effector and memory T cells without suppressing the naïve T cell population below control levels or compromising their ability to respond to a proliferative stimulus.

DISCUSSION

The presence of queuine in position 34 of $G_{34}U_{35}N_{36}$ tRNA is a hallmark of almost all eubacteria and eukarya. Despite extensive investigation, no definitive picture has emerged of the physiological purpose of this modification that has been shown to be non-essential in multiple organisms (5). Given that the effects of queuine insertion are phenotypically subtle, it is remarkable that a novel, structurally related TGT substrate (NPPDAG) causes an unprecedented, rapid and complete remission of ongoing disease in a chronic EAE model, that is TGT-dependent. This occurs without sacrificing the level and response of the naïve T cell population. Since the modification of tRNA with queuine is irreversible (6), NPPDAG can only be incorporated into the anticodon loop of tRNA that has not previously undergone the base-exchange reaction with queuine. This, coupled with multiple observations in the literature that rapidly proliferating cells are hypomodified at position 34, results in a therapeutically intriguing scenario from a selectivity standpoint: eukaryotic TGT can be exploited as a delivery mechanism for NPPDAG in rapidly expanding cell populations, including the disease-relevant encephalithogenic T cells, while ignoring tRNA in already queuine-modified adult, terminally differentiated cells. In addition to targeting cells selectively, the fact that the modification of tRNA *via* base-exchange is mechanistically unique in eukaryotic organisms

([5](#)) and that queuine and its nucleic acid derivatives are the only 7-deazaguanine compounds found naturally in humans (which are not known to be substrates for any other enzymes or to serve any other distinctive purpose) may allow one to aspire towards a level of cellular and molecular precision uncommon in nucleobase-inspired/mimicking drugs for eukaryotic targets.

As is the case with queuine itself, why the modification of selected tRNA molecules with NPPDAG leads to disease remission is currently unknown, however, the therapeutic effect unambiguously hinges on the eukaryotic TGT enzyme; as the compound is inactive in organisms deficient in TGT activity. What appears certain however, is that the exploitation of TGT—an enzyme long thought to be of marginal physiological impact—to modify tRNA is a promising avenue of investigation for the treatment of autoimmune disease.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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