Eukaryotic tRNA-guanine transglycosylase (TGT) – an enzyme recently recognised to be of potential therapeutic importance – catalyses base-exchange of guanine for queuine at the wobble position of tRNAs associated with 4 amino acids via a distinct mechanism to that reported for its eubacterial homologue. The presence of queuine is unequivocally required as a trigger for reaction between the enzyme and tRNA and exhibits cooperativity not seen using guanine as a substrate.

The post-transcriptional modification of tRNA is a powerful and labyrinthine set of tools employed by organisms to fine-tune the process of protein translation; and is being recognised as a process widely implicated in human disease. Arguably one of the most remarkable of the 100+ known such modifications is the associated with histidine, asparagine, tyrosine and aspartic acid (TGT, or have been postulated) to be characterised by tRNA hypomodification. Despite the ubiquity of this type of base-exchange (conserved in one form or another across the three kingdoms) in the anticodon loop of tRNA, the precise roles of tRNA, which also has been proposed to also serve as a queuine salvage mechanism to that reported for its eubacterial homologue. The presence of queuine is unequivocally required as a trigger for reaction between the enzyme and tRNA and exhibits cooperativity not seen using guanine as a substrate.

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The eukaryotic TGT would accept the de novo-designed synthetic nucleobase 7 (Fig. 1A) as a substrate and catalyse its displacement of 2 from unmodified tRNA. Treatment of mice given a murine model of Multiple Sclerosis – a crippling disease affecting 2.3 million people worldwide – with 7 led to an immunomodulatory effect and the complete (and unprecedented) remission of symptoms – without discernible immunosuppression. Mice in which the gene encoding for TGT had been deleted were refractory to therapy. TGT and the base-exchange it catalyses (previously often considered little more than mechanistic curiosities) are thus highly attractive targets for autoimmune disease drug development; and a greater understanding of its mode of action is highly desirable.

Eukaryotic TGT comprises a ‘heterodimer’ of a catalytically competent QTRT1 subunit and a homologous protein QTRT2, which also has been proposed to also serve as a queuine salvage enzyme, while in eubacteria, TGT is a homodimer. Garcia et al. (Fig. 1B) reported a ‘ping-pong’ reaction mechanism wherein the eubacterial TGT first binds tRNA 4 to the enzyme-substrate complex. Excision of 2 and reaction of an aspartic acid residue with the ribosyl unit of a suitable tRNA 4 at the wobble position affords the enzyme-tRNA covalent intermediate. Subsequent binding of 6 generates complex 11, which undergoes base-exchange via the pyrrolo-N-atom of 6 to give the modified tRNA 12; while releasing 8. While the idea that a protein would first interrogate and then cleave a nucleobase from multiple tRNAs without the intended replacement base being immediately present (in the absence of 6 non-productive and
reversible exchange of 2 for 2 occurs) is striking in terms of the potential risk of tRNA inactivation, this is somewhat mitigated perhaps by the fact that eubacteria synthesise 6; and thus its levels can be presumably relied upon by the cell.

No analogous studies have been carried out on eukaryotic TGT (3); its mode of action is usually assumed to be analogous to 8. We hypothesised that the aforementioned hydrolytic risk in an environment where 1 is sourced exogenously must be graver for the cell. In light of this, and the recently discovered medicinal potential of the reaction, we set out to discern the mechanism of base-exchange catalysed by 3 (Fig. 1C). Herein we report – in stark contrast to eubacterial TGT 8 – that the eukaryotic 3 operates via a sequential ‘bi–bi’ mechanism in which it first binds 1 (to give 13), serving as a trigger for subsequent binding of 4 to yield 14. Only then, with all components bound, does base-cleavage occur leading to covalent intermediate 15, which collapses to queuine-modified tRNA 5 and free enzyme.

We began by using in vitro single turnover assays involving synthesised human tyrosyl tRNA in which the guanine at position 34 has been tritiated (i.e. 4tyr-234*) and stoichiometric levels of the eukaryotic TGT enzyme in the absence of added 1 or 2 (Scheme 1). At physiological pH and temperature and a relatively high concentration of 10 μM, no excision of 234* was detected. In contrast, when the reaction is repeated in the presence of either added 1 or 2 (5.0 eq.), quantitative base-exchange occurs (as quantified by the levels of radioactivity remaining in 4tyr after reaction). Thus, the enzyme cannot operate (in an in vitro setting) via the ‘ping-pong’ mechanism associated with the eubacterial enzyme – as the presence of added nucleobase is unambiguously required for the transformation to proceed.

It was next necessary to establish if (as is the case involving 8) eukaryotic TGT catalyses base-exchange via a discrete covalent intermediate (Scheme 2). The pyrrolopyrimidine 9-deazaguanine (16) is a useful tool for this purpose, as it has been shown to bind to eubacterial TGT yet is devoid of the pyrrolo N–H moiety required for base-exchange to occur. Accordingly, we carried out an EMSA assay (see ESI†) under catalytic conditions. When 4tyr was exposed to eukaryotic TGT (1 mol% relative to 4tyr) and 16 (in small excess relative to 4tyr) followed by incubation for 60 min at 37 °C, subsequent separation of the protein-based components on an SDS-page gel led to the formation of a new band 25 kDa heavier than TGT and a decrease in the intensity of the band associated with TGT itself. This is consistent with the formation of 17. Treatment of 17 with either NaOH (aq. 100 mM, 2 min, 37 °C) or 1 (10 μM; 1 eq.) led to the reformation of the free enzyme.
Having established both that eukaryotic TGT catalyses base exchange \textit{via} a covalent intermediate (Scheme 2), and that binding of the nucleobase must precede intermediate formation (Scheme 1); we wished to ascertain which component (\textit{i.e.} the nucleobase or 4\textsuperscript{Pr}) binds to eukaryotic TGT first using initial velocity studies. The production of 4\textsuperscript{Pr} \textit{(i.e.} tRNA radiolabelled with 2\textsuperscript{*} at position 34) from the reaction between 4\textsuperscript{Pr} and 2\textsuperscript{*} catalysed by eukaryotic TGT was carried out at varying concentrations of 2\textsuperscript{*} (0.1–0.4 μM) and set fixed concentrations of 4\textsuperscript{Pr} (1–4 μM). The results of these experiments are plotted as 1/2\textsuperscript{*} vs. 1/v, where v = the initial rate in pmol min\textsuperscript{-1} (Fig. 2A). The first striking feature of this double reciprocal plot is that one does not obtain the parallel lines one would expect in a ping-pong mechanism, which further rules out such a catalytic mode of action. Instead, the plots intercept the y-axis at different values of 1/v but are characterised by distinct slopes. When the experiments are repeated, except 4\textsuperscript{Pr} is varied and then the reaction run at different concentrations of 2\textsuperscript{*}, plots that coalesce at the same y-intercept near the origin are obtained (Fig. 2B). When the primary plot in Fig. 2A is replotted as the slope of the lines associated with the different fixed concentrations of 4\textsuperscript{Pr} vs. 1/4\textsuperscript{Pr}, the resulting secondary plot passes through the origin (Fig. 2C), while a similar plot involving the intercept vs. 1/4\textsuperscript{Pr} intercepts at close to 1 (Fig. 2D). A similar slope vs. 1/2\textsuperscript{*} secondary plot (Fig. 2E) associated with the primary plot shown in Fig. 2B (where 4\textsuperscript{Pr} is varied at fixed 2\textsuperscript{*}) does not pass through the origin. These data are consistent with an ordered sequential bi–bi mechanism. In such plots, the reaction component to bind first is the one that, when its concentration is varied, has a secondary slope plot that passes through the origin (\textit{i.e.} Fig. 2C) and a primary plot when fixed with a common 1/V\textsubscript{max} intercept (\textit{i.e.} Fig. 2B). The component which binds second is that which, when its concentration is varied, the primary plot intercepts on the y-axis near the origin (Fig. 2B).\textsuperscript{13} Thus, guanine (2) binds initially, followed by sequestration of 4\textsuperscript{Pr}.

Interestingly, the corresponding treatment involving radiolabelled queuine (1\textsuperscript{*}) is more complicated.\textsuperscript{14} While a plot of v vs. 2\textsuperscript{*} exhibits standard Michaelis–Menton behaviour (Fig. 3A); use of 1\textsuperscript{*} leads to a sigmoidal plot often indicative of cooperative binding (Fig. 3B).

To investigate the origins of this phenomenon we carried out an equilibrium dialysis study (Scheme 3). Eukaryotic TGT was equipped with a polyhistidine (His) tag at the N-terminus (\textit{i.e.} 18) and exposed to excess (up to 4 μM, 40 eq.) of either 1\textsuperscript{*} or 2\textsuperscript{*} in a buffer solution optimised for maximal enzymatic activity for 3 h at 37 °C.\textsuperscript{15}

Subsequent addition of HIS-select\textsuperscript{16} Ni-based magnetic beads allowed rapid physical separation of the deazaguanine derivative-bound enzyme from unbound components through exposure to an external magnet and decantation. The radioactivity of both magnetic and solution-based components allowed the quantification of both bound and unbound 1\textsuperscript{*} and 2\textsuperscript{*}. The results of these studies are plotted in Fig. 4. A plot of the ratio of bound/unbound enzyme \textit{versus} the concentration of free deazaguanine derivative (Fig. 4A) revealed that while at a concentration of 4 μM almost the entire amount (ca. 93%) of enzyme is bound to 1\textsuperscript{*}, only approximately 48% is bound to 2\textsuperscript{*} – with very little change observed between 2 and 4 μM. Clearly, the enzyme is able to bind almost twice as much 1\textsuperscript{*} as 2\textsuperscript{*} under saturating conditions. This is an unexpected observation; apart from the irreversible nature of the base-insertion reaction, to the best of our knowledge a divergence in behaviour of this magnitude between queuine and guanine has not been reported in the literature. Together with the sigmoidal Michaelis–Menton

\begin{figure*}[ht]
\centering
\includegraphics[width=\textwidth]{Fig_2}
\caption{Initial rate studies: primary and secondary plots.}
\end{figure*}

\begin{figure*}[ht]
\centering
\includegraphics[width=\textwidth]{Scheme_3}
\caption{Equilibrium dialysis: methodology.}
\end{figure*}
which does not occur in the sole presence of the enzyme) may facilitate a second binding event in a fashion catalytically active heterodimer. This hypothesis is supported by plots of the equilibrium dialysis data involving the incorporation of radiolabelled guanine (Fig. 4B) and queuine (Fig. 4C), which show that the former nucleobase exhibits standard behaviour on Y (bound/total enzyme) versus ligand graphs yielding a linear plot, while the same experiments carried out with 1* give rise to a convex plot characteristic of positive cooperativity or ‘autoactivation’ kinetics. In summary, it has been shown that the ping-pong mechanism by which eubacterial TGT has been reported to operate is unambiguously not a feature of the analogous eukaryotic enzyme. Rather, the enzyme mediates catalysis (in a general sense) via ordered sequential bi–bi mechanism. In the absence of nucleobase, no reaction between the enzyme and tRNA is discernible from single-turnover studies. In the presence of either guanine or queuine, base-exchange occurs, and in the presence of 9-deazaguanine (a guanine mimic which can bind to TGT but not participate in base-exchange) a new species 25 kDa (the mass of the tRNA) heavier than the enzyme is detectable – consistent with the formation of a covalent intermediate between the enzyme and tRNA. Initial velocity studies demonstrated that the nucleobase binds to TGT before tRNA, and that a discrepancy in behaviour between guanine and queuine exists – principally centred on cooperativity associated with queuine not observed using guanine. This was confirmed using equilibrium dialysis experiments which showed both that the enzyme binds approximately twice as much queuine as guanine under saturation conditions, and that the binding profile involving queuine (but not guanine) is strongly suggestive of positive cooperativity. The precise mechanism of this intriguing dichotomous behaviour is unclear at this stage – experiments to elucidate this are underway in our laboratories.

Fig. 4 Equilibrium dialysis binding data for 1 and 2 to 3.

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Conflicts of interest
There are no conflicts to declare.

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