Use of a molecular beacon based fluorescent method for assaying uracil DNA glycosylase (Ung) activity and inhibitor screening

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ABSTRACT

Uracil DNA glycosylases are an important class of enzymes that hydrolyze the N-glycosidic bond between the uracil base and the deoxyribose sugar to initiate uracil excision repair. Uracil may arise in DNA either because of its direct incorporation (against A in the template) or because of cytosine deamination. Mycobacteria with G, C rich genomes are inherently at high risk of cytosine deamination. Uracil DNA glycosylase activity is thus important for the survival of mycobacteria. A limitation in evaluating the druggability of this enzyme, however, is the absence of a rapid assay to evaluate catalytic activity that can be scaled for medium to high-throughput screening of inhibitors. Here we report a fluorescence-based method to assay uracil DNA glycosylase activity. A hairpin DNA oligomer with a fluorophore at its 5′ end and a quencher at its 3′ ends was designed incorporating five consecutive U:A base pairs immediately after the first base pair (5′ C:G 3′) at the top of the hairpin stem. Enzyme assays performed using this fluorescent substrate were seen to be highly sensitive thus enabling investigation of the real time kinetics of uracil excision. Here we present data that demonstrate the feasibility of using this assay to screen for inhibitors of Mycobacterium tuberculosis uracil DNA glycosylase. We note that this assay is suitable for high-throughput screening of compound libraries for uracil DNA glycosylase inhibitors.

1. Introduction

Uracils are occasionally found in DNA because of the deamination of the resident cytosines or the direct incorporation of dUMP during replication [1–3]. Uracil DNA glycosylases (UDGs) recognize and excise the uracil base from DNA to initiate the base excision repair pathway. Of the six families of UDGs [4], family I UDGs (Ung), are evolutionarily conserved, and highly specific for excision of uracils from both the single stranded and double stranded DNAs [4–9].

Mycobacterium tuberculosis Ung (MtuUng) plays a crucial role in maintaining the integrity of its genome, which is rendered vulnerable to cytosine deaminations not only because it is G, C rich but also because of the exposure of the pathogen to RNI and ROI discharges by the host macrophages [3,10–12]. Ung removes uracil by cleaving the N-glycosidic bond between the uracil base and the deoxyribose sugar phosphate backbone generating an apurinic/apyrimidinic (AP) site which is further acted upon by the downstream repair enzymes [1,3,13–16]. MtuUng has been reported to be crucial for in vivo growth of M. tuberculosis [17]. The function of MtuUng makes it important to screen for inhibitors for various biochemical studies and growth interventions. With similar objectives, the inhibitors of DNA ligase of Haemophilus influenza, Staphylococcus aureus and Streptococcus pneumoniae; or DNA polymerase III of M. tuberculosis were deemed useful [18,19].

Ung activity assays were originally carried out using 3H uracil containing DNA as a substrate followed by the chromatography of the reaction products on anion exchange columns [20,21]. The free uracil (having no charge) elutes in the flowthrough and is quantified by scintillation counting. While this method provides a direct assay for Ung activity, it is cumbersome especially when a large set of reactions are to be analysed either for determining enzyme kinetics parameters or for setting up inhibitor screens. Subsequently, uracil containing DNA oligomer-based assays were developed [22–24] where the end labelled (radioactively or with a fluorescent probe) DNA oligomers are used in the reactions with Ung. In this assay, excision of uracil leaves behind an AP-site, which is susceptible to cleavage under alkaline conditions. Following the Ung reaction, the samples are treated with alkali and separated on denaturing polyacrylamide gels [25,26]. The presence of a faster migrating product band shows Ung activity. While this assay has been used extensively, the requirement of alkali treatment, as a second step in the assay, makes this method non-usable for real time monitoring.
of Ung activity. To overcome such limitations and to potentially study real time kinetics of the enzyme, molecular beacon based fluorescence assays were developed [22,27]. The assays employed (FITC)-d (GCAC-TJAAAGATTAATCGCCCATGTGCNAVATGTCGCCATGTCGAAATTCTTAAGTGC)-Dabcyl [22] or 5′-6-FAM-CCACUTTTGAAATTTGAACGCATGTCGAAATTCTTAAGTGC-Dabcyl′ [27] oligomers and relied on an AP-endonuclease to cleave at the AP-sites. While this method eased the analysis of the reaction products, the use of the AP-endonuclease curtailed its versatility for the real time monitoring of uracil excision as the assay of the Ung activity is limited by the activity of the downstream enzyme (i.e. the AP-endonuclease). To overcome this limitation, subsequent improvements on the molecular beacon assays incorporated many uracils on both strands of the hairpin stem (FITC-GCAC-UUAAUUCGCCCATGTCGAAATTCTTAAGTGC-Dabcyl) [22]. Although such a design avoided the AP-endonuclease step, the authors observed that it resulted in less accurate kinetic parameters of uracil excision [22]. Subsequently, other molecular beacon-based assays were designed, which suffered from the slow kinetics of fluorescence development on account of inefficient melting of the hairpin stem harbouring the fluorophore and the quencher pair [28]. It is worth noting that inefficient stem melting also limits the potential to generate maximal difference in the fluorescence of reacted and unreacted substrate in real time and displays high background.

We had earlier shown that Ung excises uracil residues from DNA oligomers even when they are flanked by AP-sites [29]. The observation was further supported by structural determinations of DNA bound Ung. In these structures, besides making specific contacts with the uracil base, Ung was seen to interact with only the flanking phosphate residues of the sugar-phosphate backbone [30,31]. We had reported that consecutively present uracils could be efficiently excised from DNA [29]. Based on this observation, for rapid melting of the stem region in the oligomer and an improved signal to background ratio, in this study, we designed a substrate (5′-5-FAM-CUUUUUGAGCTTTTGCTCAAAAAG-BHQ-1-3′) wherein five consecutive uracil residues in the stem region of the hairpin were incorporated. Further, we reasoned that given the high turnover of the Ung class of UDGs (~800 per min [1]) excision of consecutive uracils (in contrast to the earlier design where uracils were incorporated at different sites) would least affect the macrolevel kinetic parameters of uracil excision ($K_n$ and $k_{cat}$), which are more likely to be limited by the step of the search of the uracil sites in the substrate. The hairpin oligomer was terminally attached with a commonly used fluorophore, 5-carboxyfluorescein (5-FAM) which is quenched by BHQ-1 [22,32]. Upon treatment with Ung, excision of the consecutive uracils results in unwinding of the stem region of the oligomer to rapidly separate the quencher from the fluorophore yielding an intense fluorescence signal. We demonstrate the sensitivity of this method and its application in determining the efficiency of inhibition of MtuUng by uracil derivatives [33].

2. Materials and methods

2.1. MtuUng purification

The MtuUng expression vector [34] was introduced into E. coli BL21 (DE3) Rosetta by the calcium chloride method of transformation [35]. A loopful of freshly obtained transformants were inoculated into 100 ml Luria–Bertani medium (LB), grown overnight and sub-cultured into 5.2 L LB containing 0.1 mg/ml ampicillin and 0.03 mg/ml chloramphenicol. The cultures were incubated at 24 °C for 36 h. Cells were harvested by centrifugation at 4 °C and resuspended in 15 ml 10 mM Tris–HCl (pH 8) containing 10% (v/v) glycerol, 500 mM NaCl and 1 mM phenyl methyl sulfonyl fluoride (PMSF), disrupted by sonication and spun in an SW-41Ti rotor at 25,000 rpm for 60 min at 4 °C. The supernatant was loaded onto a pre-equilibrated Ni–NTA column (5 ml) and eluted with imidazole gradient (0–500 mM) in the above mentioned buffer using FPLC. Fractions enriched for MtuUng were loaded on to Sephadex-G75 column, and eluted with a buffer containing 10 mM Tris–HCl (pH 8), 10% glycerol, 500 mM NaCl and 2 mM β-mercaptoethanol. The fractions enriched for MtuUng were pooled and loaded onto Ni–NTA column (1 ml), eluted with 280 mM imidazole, dialyzed against 20 mM Tris–HCl (pH 8), 10% glycerol, 2 mM β-mercaptoethanol and 50 mM NaCl, estimated by Bradford’s method using bovine serum albumin (BSA) as standard, and stored at −20 °C [36].

2.2. DNA oligomer and Ugi

A hairpin DNA oligomer, containing five consecutive uracils (5′-CUUUUUGAGCTTTTGCTCAAAAAG-3′) and modified at the 5′ and 3′ ends by fluorescein (5-FAM) and black hole quencher (BHQ1) respectively, was obtained from Macrogen, Inc, S. Korea following HPLC purification, resuspended in water (Milli Q), to a concentration of 100 μM. The excitation and emission wavelengths of 5-FAM are 496 nm and 520 nm.

| No. | Inhibitor         | Structure                  | Docking Score | $IC_{50}$ in mM (mean ± SEM) |
|-----|------------------|----------------------------|---------------|-------------------------------|
| 1   | Uracil           | O                           | −10.268       | 2.05 ± 0.3                    |
| 2   | 4-Thio-Uracil    | O′                          | −4.806        | 3.57 ± 0.35                   |
| 3   | 5-Iodo-Uracil    | O′                          | −9.103        | 4.92 ± 0.51                   |
| 4   | 5-Chloro-Uracil  | O                           | −8.805        | 8.71 ± 0.70                   |
| 5   | 5-Fluoro-Uracil  | O′                          | −8.963        | 50.18 ± 3.85                  |
| 6   | 5-Nitro-Uracil   | O′                          | −5.054        | 10.77 ± 0.32                  |
| 7   | 5-Amino-Uracil   | O                           | −9.636        | 7.18 ± 0.97                   |
| 8   | 5-Bromo-Uracil   | O′                          | −8.733        | 13.59 ± 0.73                  |
| 9   | Barbituric acid  | O                           | −12.03        | 7.29 ± 0.32                   |

Table 1 List of compounds screened with Dose-dependent inhibition analysis: All the compounds were treated with 0.3 x 10⁻⁶ M of MtuUng followed by addition of 30 x 10⁻⁶ M of the fluorescence -labelled oligomer. The reaction volume was taken to be 100 μl buffer by 50 mM Tris-Cl (pH 8), 1 mM Na₂EDTA and 37.59 nM BSA (n = 3).
nm, respectively, whereas the absorbance maxima of BHQ-1 is 534 nm.

Ugi was used from the lab stock purified [37] from an overexpression construct of PBS1 phage encoded Ugi [38].

2.3. Compounds for inhibitor screening

The uracil derivatives selected for analyzing their potential for MtuUng activity inhibition (Table 1) were obtained from Sigma-Aldrich [33,39] and dissolved in 100% DMSO to prepare 100 mM stock solutions.
2.4. Assays for Ung activity and inhibition using the fluorescence assay

The reactions (100 μl) were carried out in a Corning clear-bottom 96 well black plates. The concentrations of the oligomer, MtuUng and Ugi were standardised to 30 nM, 0.3 nM and 2.1 nM, respectively. The reaction was carried out in a buffer containing 50 mM Tris-Cl (pH 8), 1 mM Na₂EDTA and 37.6 nM BSA. A control reaction with 80 mM NaOH (in place of MtuUng) was included to monitor melting of the oligomer. After 1.5 mm amplitude of orbital shaking of 10 s, the fluorescence was quantified every 2 min for 40 min in bottom reading mode at 37 °C, with 495 nm as excitation wavelength and 520 nm as emission wavelength using Tecan infinite 200Pro. The excitation and emission bandwidth were 9 nm and 20 nm respectively.

2.5. Gel based method to monitor MtuUng activity on uracil containing substrate

The reactions (5 μl) set up as above, consisted of 6 nM MtuUng, 42 nM Ugi (when required) followed by the addition of 100 nM oligomer. The reactions were incubated at 37 °C for 40 min, treated or not treated with and 0.096 N NaOH at 95 °C for 10 min, mixed with loading buffer (0.5 mM Na₂EDTA, 64% formamide) and analysed by electrophoresis on 20% polyacrylamide (19:1 cross-linking) and on Sapphire Biomolecular Imager at 488 nm.

2.6. Graphical analysis

All the graphs were plotted and analysed using GraphPad Prism Version 5.

3. Results and discussion

3.1. Development of fluorescence-based assay to analyse uracil excision by MtuUng

A 24-mer hairpin DNA oligomer containing five consecutive uracil residues in its stem, incorporated immediately after the cytosine at the 5’ end, was used as substrate. The oligomer has a high quantum yield fluorophore, 5-carboxyfluorescein attached with cytosine at the 5’ end, was used as substrate. The oligomer has a high quantum yield fluorophore, 5-carboxyfluorescein attached with cytosine at the 5’ end, was used as substrate. The oligomer has a high quantum yield fluorophore, 5-carboxyfluorescein attached with cytosine at the 5’ end, was used as substrate. The oligomer has a high quantum yield fluorophore, 5-carboxyfluorescein attached with cytosine at the 5’ end, was used as substrate. The oligomer has a high quantum yield fluorophore, 5-carboxyfluorescein attached with cytosine at the 5’ end, was used as substrate. The oligomer has a high quantum yield fluorophore, 5-carboxyfluorescein attached with cytosine at the 5’ end, was used as substrate. The oligomer has a high quantum yield fluorophore, 5-carboxyfluorescein attached with cytosine at the 5’ end, was used as substrate. The oligomer has a high quantum yield fluorophore, 5-carboxyfluorescein attached with cytosine at the 5’ end, was used as substrate. The oligomer has a high quantum yield fluorophore, 5-carboxyfluorescein attached with cytosine at the 5’ end, was used as substrate.

![Fig. 2. Determination of kinetic parameters of uracil excision by MtuUng: (A) Background subtracted continuous graph showing linear relationship between product formation and substrate concentration in the presence of MtuUng. The enzyme concentration of 0.3 x 10⁻⁹ M was used with the substrate concentrations ranging from 40 x 10⁻⁹ to 640 x 10⁻⁹ M, n = 3. (B) Graph of the reaction velocities, calculated as the slopes of the data from [A], as a function of the oligomer concentration depicting the Michaelis-Menten trend. (C) The Lineweaver-Burk graph from data in [B] with inverse range of substrate concentration from 40x10⁻⁹-640x10⁻⁹ M to determine K_m and V_max. n = 3, R² = 0.97.](image-url)
in lane 7 [29]. The faint bands seen towards the end of the gel in lanes 6–8 most likely represent the free FAM generated by its cleavage from the oligomer because of treatment under alkaline conditions at high temperature of 95 °C. In fact, in these lanes we see some background bands (migrating between the substrate and product bands) which could be due to damage to the integrity of the stem loop structure of the oligomer when heated at 95 °C in the presence of NaOH. Nonetheless, the analyses in Fig. 1C further support the oligomer design and show that conventional treatment of the oligomer with NaOH at high temperature is not required to generate high signal to noise ratio of the fluorescence for Ung assays. Importantly, the treatment of the oligomer with NaOH at 37 °C does not show any degradation products (lane 5) suggesting that the fluorescence enhancement in the NaOH treated reaction in Fig. 1B, is due to the melting of the oligomer.

3.2. Assay of MtuUng activity and determination of kinetic parameters of uracil excision

To check the sensitivity of this assay, we performed an experiment with different concentrations of the substrate against a fixed enzyme concentration which showed direct relationship between fluorescence intensity and substrate concentration (Fig. 2A). The hyperbolic curve of velocity ($v_o$) with respect to the substrate concentration [S] explained the velocity dependence on the substrate concentration (Fig. 2B) [45].

To avoid systemic errors, $K_m$ and $V_{max}$ ($k_{cat}$) were calculated from the non-linear regression curve and plotted on double reciprocal graph (1/$v_o$ versus 1/[S]) to check goodness of fit (Fig. 2C). The double reciprocal graph yielded a straight line with $R^2$ of 0.97 (the Lineweaver-Burk plot). $K_m$ derived from this method (152.3 ± 0.2 nM) is comparable to those determined earlier for MtuUng or other Ung proteins [46–48], and the calculated $k_{cat}$ was 0.38 ± 0.03 s$^{-1}$. In addition, the results confirm the uracil excision being first order reaction with respect to MtuUng concentration.

3.3. Analysis of inhibitory effect of uracil derivatives on MtuUng activity

The products formed from the MtuUng action on uracil containing DNA are known to act as its inhibitors [1]. The product, uracil, binds to the enzyme and acts as its inhibitor [1,49]. To show the utility of the method and to investigate their efficiencies towards MtuUng, different uracil derivatives were screened to bind to MtuUng in terms of
thermodynamic parameters [33] and computational analysis with measure of docking score using Schrödinger Software (Table 1). In addition, bituric acid was checked owing to its high similarity to the uracil ring and its general applicability in inhibitor design for a variety of targets [50–53]. The selected compounds were analysed for dose dependent inhibition. The graphs of fluorescence produced as a function of inhibitor concentration showed an IC50 (half-maximal inhibitory concentration) value which was taken as a measure of inhibitor efficiency (Table 1). The IC50 of uracil derived from this method is 2.05 ± 0.3 mM (Fig. 3A and B).

3.4. Application of the method in determination of inhibition kinetics

We could further exploit the real time detection of this method by understanding type of inhibition the enzyme undergoes by graphically plotting initial velocity of MtuUng in the presence of the inhibitor as a function of substrate concentration. For instance, one of the uracil derivatives, 5-chloro-uracil was checked by plotting such graphs (Fig. 4A). The Lineweaver-Burk plot of the same data showed conversion point of different inhibitor concentrations above the x-axis and to the left of y-axis, signifying linear mixed inhibition (Fig. 4B) [54]. The data reported earlier [1,55–59] had also suggested the a non-competitive mode of inhibition by uracil. In addition, the inhibitor efficiency was explored by means of Ki (inhibitory constant) [45]. To obtain values of KI of 5-chloro-uracil, secondary graph was plotted. The slope (Fig. 4A) as a function of inhibitor concentration showed a value of KI (intercept on x axis) as 7.036 mM (Fig. 4B).

4. Conclusions

The novelty of this method is defined by a single reaction, real time analysis of an enzyme (Ung) action on DNA. Unlike other methods, requirement of minimal constituents and quick results from the assay ensure speedy and reliable analysis of comparatively complex enzyme kinetics. The one step analysis is supported by the design of substrate since incorporation of multiple consecutive uracils in the oligomer eliminates the need for thermal or pH stimuli to facilitate the opening of the hairpin stem of the DNA oligomer to separate the fluorophore and the quencher. Moreover, the sensitivity of 5 nM difference in substrate concentration shows the method as highly promising for detecting the in vitro efficiency of several biological enzymes acting on DNA damage. The inhibition analysis by this method, endorses high-throughput screening of compounds which can accelerate the process of drug discovery against infectious diseases by targeting their DNA associated proteins.

Declaration of competing interest

The authors declare no conflicts of interest.

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