In Vitro Fluorogenic Real-Time Assay of the Repair of Oxidative DNA Damage


The repair of oxidative damage to DNA is essential to avoid mutations that lead to cancer. Oxidized DNA bases, such as 8-oxoguanine, are a main source of these mutations, and the enzyme 8-oxoguanine glycosylase 1 (OGG1) is the chief human enzyme that excises 8-oxoguanine from DNA. The activity of OGG1 has been linked to human inflammation responses and to cancer, and researchers are beginning to search for inhibitors of the enzyme. However, measuring the activity of the enzyme typically requires laborious gel-based measurements of radiolabeled DNAs. Here we report the design and properties of fluorogenic probes that directly report on the activity of OGG1 (and its bacterial homolog OPG1) in real time as the oxidized base is excised. The probes are short, modified DNA oligomers containing fluorescent DNA bases and are designed to utilize 8-oxoguanine itself as a fluorescence quencher. Screening of combinations of fluorophores and 8-oxoguanine revealed two fluorophores, pyrene and tCo, that are strongly quenched by the damaged base. We tested 42 potential probes containing these fluorophores: the optimum probe, OGR1, yields a 60-fold light-up signal in vitro with OGG1 and Fpg. It can report on oxidative repair activity in mammalian cell lysate and with bacterial cells overexpressing a repair enzyme. Such probes might prove useful in quantifying enzyme activity and performing competitive inhibition assays.

Introduction

8-Oxoguanine is an abundant form of oxidative DNA damage that is generated by reactive oxygen species resulting from metabolism, ionizing radiation, and chemicals.[1–5] 8-Oxoguanine in DNA can adopt a syn conformation and pair with adenine during replication,[6,7] ultimately leading to G-to-T mutations.[8–10] This form of nucleobase damage might be the chief molecular source of oxidation-induced mutagenesis in cells.[11]

As protection against the mutagenic potential of 8-oxoguanine, cells have evolved multiple ways to repair this lesion. One of the chief eukaryotic 8-oxoguanine repair enzymes is 8-oxoguanine glycosylase 1 (OGG1), which preferentially removes 8-oxoguanine opposite cytosine in double-stranded DNA.[12, 13] OGG1 acts as a glycosylase to excise the 8-oxoguanine base. OGG1 can then act as an AP lyase to perform a β-elimination reaction on the 3′-side of the abasic site to generate an α,β-unsaturated aldehyde at the 3′ terminus.[12, 13] However, OGG1’s lyase activity proceeds slowly and is believed not to be relevant in vivo; it has been proposed that the endonuclease APE1 instead cleaves the DNA backbone following 8-oxoguanine excision by OGG1.[14–18] Formamidopyrimidine-DNA glycosylase (Fpg), the functional homologue of OGG1 in Escherichia coli, excises the 8-oxoguanine base and then performs β and δ elimination reactions at the abasic site to generate a single nucleotide gap flanked by 3′- and 5′-phosphates.[19–20] Both OGG1 and Fpg prefer double-stranded DNA substrates, but Fpg can also act on single-stranded DNA.[21, 22]

E. coli, Saccharomyces cerevisiae, and mice engineered to be deficient in Fpg or OGG1 display increased levels of genomic 8-oxoguanine and increased mutations, thus highlighting the importance of these enzymes in maintaining genome integrity.[26–30] OGG1 mutants with decreased or abolished 8-oxoguanine repair activity have been found in many cancer specimens,[31–39] and lower OGG1 activity has been associated with increased cancer risk.[40–41]

In addition to its prominent association with genetic mutations leading to malignancies, altered OGG1 activity has been associated with many other important diseases. Several OGG1 mutants with decreased or abolished activity have been discovered in Alzheimer’s patients.[44, 45] Two OGG1 polymorphisms have been associated with rheumatoid arthritis,[46] and decreased OGG1 activity has been linked with decreased lung inflammation in an allergy model.[47] Finally, altered levels of OGG1 have been associated with type 2 diabetes.[48, 49]

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A simple method to measure OGG1 activity would help further elucidate these multiple links between OGG1 activity and disease. Such a method would also aid screening for OGG1 inhibitors or activators, which would be useful as drugs and research tools, and assist determining disease prognosis from patient samples. Currently, quick and easy study of OGG1 activity is hindered by the time-consuming and indirect nature of available methods (e.g., PAGE, comet assay, GC/MS, ELISA, HPLC, and qRT-PCR). For example, the strand-nicking assay (one of the most common methods used to measure OGG1 activity) involves radiolabeling an 8-oxoguanine-containing oligonucleotide, incubating it with the sample of interest, and analyzing the sample at various time points by gel electrophoresis and radioactivity measurement. This method requires at least a day and only measures OGG1 activity at discrete, predetermined times. More recently developed approaches using fluorescently labeled oligonucleotides give a fluorescent readout of OGG1 activity, but these probes rely on backbone cleavage (which is done slowly by OGG1, and is believed to be performed by a separate enzyme in human cells) and subsequent DNA dehybridization to generate a fluorescence signal. Thus, these FRET-based probes do not directly measure the excision of 8-oxoguanine but instead rely on downstream events following the excision. Other DNA-cleaving and DNA-unwinding activities (by various nuclease and helicase) can yield false signals with this design. A split-luciferase sensor that detects 8-oxoguanine in DNA could potentially be used to identify competitive inhibitors of OGG1 or to indirectly measure OGG1 activity, but this sensor measures the binding of OGG1 to 8-oxoguanine rather than OGG1 enzymatic activity.

Given the need for a more direct and simple measurement of 8-oxoguanine excision activity, we aimed to develop "light-up" probes that directly measure excision of this oxidized base in real time. By identifying and applying a fluorophore that is strongly quenched by 8-oxoguanine, we have developed a probe that gives a 60-fold fluorescence increase upon reaction with OGG1 or Fpg. Such probes enable direct and specific measurements of OGG1 and Fpg activity in vitro and are expected to be useful in screening polymorphisms, mutations, post-translational modifications, and competitive inhibitors of 8-oxoguanine repair enzyme activity.

Results and Discussion

Probe design

Our probe design relies on a fluorophore that is quenched specifically by 8-oxoguanine, but becomes emissive following excision of the damaged base. Most desirable would be a fluorophore that is not strongly quenched by native (undamaged) nucleobases, so that a DNA enzyme-binding sequence can be constructed while retaining efficient fluorescence emission. Although the "8-0xo-G clamp" is known to be quenched by 8-oxoguanine, this nucleobase analogue pairs with and surrounds the damaged base, and thus might prevent recognition or excision by repair enzymes. To screen for fluorophores that are quenched by 8-oxoguanine, we assembled dinucleotides consisting of a fluorophore deoxyribose (a nucleoside with a fluorophore replacing the nucleobase) and either 8-oxoguanine or an abasic site mimic (representing the product following 8-oxoguanine excision). (Structures of the dye monomers and a representative dinucleotide are shown in Figure S1 in the Supporting Information.) By comparing the fluorescence emission intensities of the 8-oxoguanine-containing dinucleotides with those of the abasic controls, we found that the fluorophores pyrene, 2-aminopurine, ethenoadenine, pyrrolo-dC, and tCo were quenched two- to 30-fold by 8-oxoguanine, with pyrene and tCo most strongly quenched (Figure 1). Pyrene has been reported to be quenched strongly by thymine and cytosine, and moderately by guanine. Similarly to a previous report of quenching of tCo by guanine, tCo was more strongly quenched by 8-oxoguanine on the 5'-side of the fluorophore than on its 3'-side. Having identified at least two dyes that are strongly quenched by 8-oxoguanine in DNA, we synthesized 42 candidate probes as putative enzymatic substrates that contain 8-oxoguanine and one or more of these fluorophores in place of natural nucleobases (Figure S2). Oligonucleotide design was guided by the desire to keep probes short, in order to aid synthesis yield and enhance the likelihood of cellular uptake. Some probes were single-stranded; others were double-stranded with a hairpin design. For the latter, we chose a highly stabilizing GAA loop to enhance thermal stability in short sequences. The probes were prepared by standard phosphoramidite chemistry on an automated DNA synthesizer, purified by HPLC or PAGE, and characterized by MALDI-TOF MS to confirm incorporation of both the damaged nucleobase and the fluorescent deoxyribose. The probes were screened for increases in fluorescence emission after incubation with recombinant human OGG1 or Fpg (signal enhancements for all probes with the enzymes in Figure S3).

We observed some trends with pyrene-containing probes. Firstly, short double-stranded (hairpin) probes were much better substrates than single-stranded probes (compare OGR2-17 vs. OGR18-32 in Figure S3). Secondly, a fluorophore on the 3'-side of 8-oxoguanine appeared to be more favorable for enzymatic reaction than on the 5'-side (OGR19 vs. OGR21). To several of the pyrene-based probes, we added a second fluorophore as a FRET or exciplex partner, as a possible strategy for yielding longer-wavelength emission. In these cases, quenching by 8-oxoguanine was less efficient than for the single-fluorophore designs, giving high initial fluorescence signals. Many of the pyrene-based probes exhibited low turnover; this might be a consequence of the bulky, hydrophobic fluorophore, or the affinity of OGG1 for the abasic site included as the pyrene pairing partner. We also observed trends with tCo-containing probes. Adding a second 8-oxoguanine to the other side of tCo did not diminish the initial signal but did decrease the final signal (OGR1 vs. OGR36). MALDI-TOF MS analysis (data not shown)
Figure 1. Fluorescence quenching (emission spectra) of various fluorophores by 8-oxoguanine. DNA dinucleotides consisted of a fluorescent nucleoside adjacent to 8-oxoguanine or an abasic site mimic. Fluorescent bases: A) 2-aminopurine, B) etheno adenine, C) perylene, D) pyrene, E) pyrrolo-dC, F) QB, G) and H) tCo, and I) V. Dinucleotides in F and G have the fluorophore on the 3'-side of 8-oxoguanine or the abasic site mimic. In all other panels the fluorophore is on the 5'-side of 8-oxoguanine or the abasic site mimic.
indicated that only one 8-oxoguanine was excised, presumably rendering the probe a poor substrate for excision of the second 8-oxoguanine and thus keeping the probe in a semi-quenched state. Increasing the stem length or removing the loop did not increase probe performance, thus indicating that the hairpin structure did not inhibit the enzyme (OGR1 vs. OGR37, OGR41, and OGR42).

**In vitro characterization**

Of the 42 probes tested, the best-performing was a short hairpin that contains the fluorescent cytosine analog tCo opposite guanine and flanked on the 5’-side by 8-oxoguanine and on the 3’-side by guanine (OGR1, Scheme 1). The hairpin structure was stable (Tm > 90 °C; Figure S4), and the probe yielded a 60-fold fluorescence increase upon reaction with Fpg (Figure 2A.)

The quantum yield of the probe (emission maximum 460 nm) was 0.008 before the reaction and 0.170 after completion. The probe reacted more quickly with Fpg than hOGG1 (reaching 90% of final fluorescence after 10 min and 3 h, respectively, for a 1:6 molar ratio of enzyme to substrate; Figure 2B). From the initial rates of increase as a function of concentration, we determined apparent Km values of 773 ± 120 nM (hOGG1) and 14.5 ± 2.9 nM (Fpg), and kcat of 0.07 and 0.2 min⁻¹, respectively.

Km has been reported as 3.4 nM (hOGG1) and 6 nM (Fpg) and kcat as 300 min⁻¹ (hOGG1) and 3 min⁻¹ (Fpg), with conventional double-stranded DNA substrates.[24,45]

Analysis of the hOGG1 reaction products by PAGE and MALDI-TOF MS identified the probe following cleavage of the DNA backbone with a phosphate at the new 5’ terminus and an abasic site at the new 3’ terminus. Analysis of the Fpg reaction products identified the probe following cleavage of the DNA backbone with phosphates at the new 3’ and 5’ termini. These observations are consistent with the known mechanisms of these enzymes (Figure 3). Thus the analysis confirms enzymatic excision of the fluorescence-quenching damaged base as the mechanism of probe response.

**Performance with bacterial cells overexpressing a repair enzyme**

Although assays with purified enzyme have general utility, the ability to directly evaluate enzyme activity in bacterial cells (without purification), would save steps in some applications. In a preliminary experiment, we used pOGR1 (a nuclease-protected variant of probe OGR1 with 2’-O-methyl groups at the termini; Figure S5) with E. coli overexpressing Fpg. Probe pOGR1 incubated with these cells in the presence of 0.25% Triton-X 100 to permeabilize the cells showed an increase in fluorescence after just 1 hour (controls: cells not overexpressing Fpg, or without pOGR1; Figure 4). The results show that the probe can be used to assess enzymatic activity directly in bacterial cell suspensions.

**Performance in mammalian cell extracts**

To test whether OGG1 activity can be detected in mammalian cell extracts, we incubated pOGR1 with extract from WT and Ogg1⁻/⁻ MEF cells[47] (Figure 5). The WT cell extract yielded higher fluorescence (~2.5 times greater than in the deleted cell line), and the reproducibility was high, with small errors relative to the signal. The difference between the two cell lines was reproducible beyond the error limits after 8 h, and became more readily apparent at longer times. We conclude that the probe can detect OGG1 activity at native levels in mammalian cell extracts.

**Utility in detecting inhibitors of OGG1**

Next we tested whether OGR1 can be used in reporter assays for competitive inhibitors of OGG1. A previous study identified the unnatural nucleobase 3-Cl-4-F-indole in double-stranded DNA as a strong binder of OGG1.[62] We synthesized a short stable hairpin containing 3-Cl-4-F-indole (sequence in Figure S6) as a test inhibitor. From the initial rate of fluorescence increase, we determined apparent Kd of OGR1 with hOGG1 to be 0.77 ± 0.12 μM in the absence and 3.71 ± 0.52 μM in the presence

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**Scheme 1.** A) Quenching and generation of fluorescence signals with base excision. 8-Oxoguanine is represented as “oG” and the fluorophore is represented as “F”. The fluorophore is quenched by 8-oxoguanine but becomes emissive following excision of 8-oxoguanine. B) Short hairpin probe OGR1 (the best-performing probe in this study) undergoes base excision by OGG1. 8-Oxoguanine is represented as G*; tCo (1,3-diaza-2-oxophenoxazine) is represented as C*.
OGG1 is known to have a strong preference for 8-oxoguanine (OGR1) in double-stranded DNA. Fpg also accepts single-stranded substrates. In our assay, the signal-to-noise ratio passed the cutoff after only 10 min, and the Z factor passed the cutoff after 25 min, thus showing that long incubation times are not needed to assess enzyme activity with the probe (coefficient of variance <20% at all times). Although greater signal intensity was achieved with OGR1 after longer reaction times, it is not necessary for the reaction to go to completion; comparison of the initial rates of fluorescence increase or of the fluorescence obtained after a specified time window would suffice. At 0.8 μM probe in a reaction volume of 20 μL, one micromole of probe (the scale of a standard DNA synthesis) would be sufficient for approximately 60,000 assays.

**Conclusion**

We have generated a fluorescence turn-on probe for 8-oxoguanine excision. It yields a 60-fold increase in fluorescence upon incubation with human OGG1 or bacterial Fpg. In this novel design, 8-oxoguanine acts as a quencher, thereby providing a direct, real-time fluorescence signal that depends on its excision from DNA. Because of this direct measurement of enzymatic bond cleavage, the probe offers advantages over FRET-based probes, which require downstream events such as DNA backbone cleavage and dehybridization to yield a fluorescent signal, as well as other common indirect and time-consuming methods to measure 8-oxoguanine excision. Compared with a previously reported FRET sensor for OGG1, our probe displays higher fluorescence fold-change, and its small size (relative to previous FRET-based probes) makes it easier and less costly to synthesize. Furthermore, the highly stable double-stranded hairpin design eliminates the need to carefully anneal the probe and obviates issues of strand stoichiometry. We expect that this and related probes will be useful for screening the effects of polymorphisms, mutations, post-translational modifications, and competitive inhibitors on 8-oxoguanine repair enzymes.

In our molecular strategy for direct measurement of DNA base excision by fluorescence, the nucleobase to be excised acts as a fluorescence quencher. Specific coupling of the damaged base with a photophysically matched fluorescent nucleobase gives a probe that yields a light-up signal in real time only when the damaged base is removed. This strategy was previously tested with uracil deoxyglycosidase, and the uracil (which arises from hydrolytic damage of cytosine) was combined with a fluorescent pyrene nucleoside to report removal of uracil. The current experiments suggest that the application of such a fluorescence-quenching strategy might be more general for measuring base excision repair. Here we have shown that it can be employed for 8-oxoguanine, by taking advantage of previously unknown quenching of tCo by the damaged base.

Fpg reacted approximately 18 times faster than human OGG1 with probe OGR1 under our conditions (Figure 2B). Whereas OGG1 is known to have a strong preference for double-stranded DNA substrates with 8-oxoguanine paired opposite a cytosine, Fpg also accepts single-stranded substrates. The higher activity of Fpg on the new probe relative to OGG1 might reflect Fpg’s broader substrate requirement. The fact that OGR1 is a significantly less efficient substrate than natural DNA for OGG1 suggests that the extra steric bulk of the fluorescent cytosine derivative tCo interacts unfavorably in the OGG1 active site. However, the probe showed useful activity in vitro and in cell lysates. In the cell-based tests, the signal was slower to develop than with pure enzyme in vitro; we attribute this to inherently low enzyme activity in these systems and to significant cellular background fluorescence at
this wavelength. Nevertheless, assays over 6–12 h ave reproducible signals of this enzyme activity.

Taken together, the results show that the OGR1 probe performs well in in vitro assays of enzyme activity with purified enzyme and with cell lysates, as well as for the measurement of activity in bacteria, which is expected to be useful for measurement of inhibition and of the effects of mutations on the enzymes. The probe gives a sufficiently high signal and low enough variation for use in high-throughput screens for competitive OGG1 inhibitors. As single DNA synthesis (1 mmol scale) would allow approximately 60 000 assays in a 384-well plate format. The fact that all components of probe OGR1 are commercially available makes it widely accessible for researchers.

**Experimental Section**

**Oligonucleotide preparation:** Adenine, thymine, guanine, cytosine, 2'-O-methylcytosine, 8-oxoguanine, spacer (tetrahydrofuran), pyrrolo-dC, 2-aminopurine, ethenoadenine, Dss, tCo phosphoramide, 1 μmol columns of dG-CPG, da-CPG, Universal Support III, and DNA synthesis reagents were purchased from Glen Research (Sterling, VA).

Figure 3. PAGE analysis of 4 μM probe OGR1 5’-labeled with [32P]phosphate and reacted with 658 nm A) hOGG1 or B) Fpg at 37 °C for the indicated times. Piperidine (200 mM) was added and incubated (95 °C, 5 min) to cleave the DNA backbone at abasic sites as indicated to identify the 8-oxoguanine excision product and the backbone cleavage product. Below: MALDI-TOF MS analysis of OGR1 following overnight reaction with C) hOGG1 or D) Fpg.

All oligonucleotides excluding OGR37–40 were synthesized on a 500 μmol scale with standard 3'-to-5' cyanoethyl phosphoramidite chemistry with 999 s coupling times for pyrene, perylene, and phenylethynylpyrene, 180 s for tCo, and 240 s for other bases. Oligonucleotides OGR37–40 were synthesized by the Peptide and Nucleic Acid Facility (Stanford University, CA). Oligonucleotides containing 8-oxoguanine were deprotected in concentrated ammonium hydroxide with 2-mercaptoethanol (0.25 M) for 17 h at 55 °C. Dinucleotides containing pyrene, perylene, or phenylethynylpyrene (but not 8-oxoguanine) were deprotected in potassium carbonate (0.05 M in methanol) for 24 h at room temperature. All other oligonucleotides were deprotected in ammonium hydroxide for 17 h at room temperature. The deprotected oligonucleotides were dried in a SpeedVac (Savant).

Oligonucleotides were purified by either HPLC or PAGE. For purification by HPLC, samples were purified on an HPLC device (Shimadzu) with a Jupiter C5 column (Phenomenex, Torrance, CA) or a Prophere C18 column (Grace, Columbia, MD); acetonitrile and triethylammonium acetate (0.1 M, pH 7) were the eluents. For purification by PAGE, crude oligonucleotide was loaded onto a 20% denaturing polyacrylamide gel (acrylamide/bis (19:1, 20%), urea (7.5 M), tris borate-EDTA buffer). The gel was cooled with a fan during electrophoresis. The gel was visualized by UV absorbance, and the darkest band was excised and extracted in NaCl (0.2 M) and 2-mercaptoethanol (0.25 M) overnight at 4 °C with shaking. The sample was dialyzed at 4 °C, filtered, dried in a SpeedVac, and resuspended in water.

Probe concentration was determined by fluorophore absorbance:

pyrene \( \varepsilon_{340} = 39 000 \text{ M}^{-1} \text{cm}^{-1} \) [67];

perylene \( \varepsilon_{340} = 39 200 \text{ M}^{-1} \text{cm}^{-1} \) [68].
2-aminopurine $e_{260} = 1000 \text{ M}^{-1} \text{ cm}^{-1}$ \cite{71} 
either adenine $e_{260} = 4800 \text{ M}^{-1} \text{ cm}^{-1}$ \cite{72} 
either pyrrolo-dC $e_{340} = 3700 \text{ M}^{-1} \text{ cm}^{-1}$ \cite{73} 
either Dss $e_{380} = 31000 \text{ M}^{-1} \text{ cm}^{-1}$ \cite{74} 
either tCo $e_{365} = 9000 \text{ M}^{-1} \text{ cm}^{-1}$ \cite{19} 
either phenylethynylpyrene $e_{365} = 67000 \text{ M}^{-1} \text{ cm}^{-1}$ \cite{19} 
either 3-Cl-4-F-indole $e_{260} = 13700 \text{ M}^{-1} \text{ cm}^{-1}$ \cite{62}

The QB extinction coefficient was calculated to be $22400 \text{ M}^{-1} \text{ cm}^{-1}$ according the Beer–Lambert Law by measuring the absorbances of known concentrations of QB diol in methanol. For oligonucleotides with multiple nucleobases or fluorophores absorbing at the specified extinction-coefficient wavelength, the extinction coefficient was calculated as the sum of the extinction coefficients of each nucleobase or fluorophore in the oligonucleotide.

Oligonucleotide identities were confirmed by MALDI-TOF MS performed at the Stanford Peptide and Nucleic Acid Facility. See Figure 4.

**Figure 4.** Reporting on 8-oxoguanine excision with bacterial cells expressing a repair enzyme. A) *E. coli* overexpressing Fpg after 17 h with 4 μM nuclease-protected probe pOGR1 and 0.25% Triton X-100 (---), overexpressing Fpg after 17 h with 0.25% Triton X-100 (---); basal Fpg expression after 17 h with 4 μM probe and 0.25% Triton X-100 (---). B) *E. coli* overexpressing Fpg with 4 μM probe and 0.25% Triton X-100 after 1 h (---), 4 h (-----), or 17 h (---).

**Figure 5.** Reporting 8-oxoguanine excision in mammalian cell extracts. Probe pOGR1 (2 μM) was incubated with 6.7 ng/μL WT (*) or Ogg1−/− (o) MEF cell extracts at 37 °C. The fluorescence at 460 nm ($λ_{ex} = 355 \text{ nm}$) was measured at the indicated time points. Background fluorescence (without probe) was subtracted. Error bars show standard deviation ($n = 5$).
Table S1 for the calculated and found masses. Samples were stored at -20 °C.

Fluorescence analysis of in vitro enzymatic activity: Fpg reaction buffer (NEBuffer 1), hOGG1 reaction buffer (NEBuffer 4), and BSA (10 mg/mL) were purchased from New England Biolabs.

The emission spectra were obtained with a Jobin Yvon Spex Fluorolog 3 spectrophotometer (Horiba, Kyoto, Japan), by exciting the sample at the fluorophore excitation maximum (pyrene 342 nm, pyrene 440 nm, 2-amino-purine 303 nm, etheno adenine 276 nm, pyrrolo-dC 347 nm, Dss 360 nm, phenylethynylpyrene 363 nm, QB 400 nm) and measuring emission starting at 10 nm above the excitation wavelength (step size 1 nm).

Aqueous solutions of 1 × Fpg or hOGG1 reaction buffer and BSA (100 µg/mL) were prepared in a quartz cuvette. The fluorescence emission spectrum was measured before addition of probe to determine buffer fluorescence. Fluorescence emission spectra were recorded immediately after addition of probe (4 µM) and after 10 min of incubation at 37 °C (to confirm stable fluorescence upon heating). For OGR1 studies, 658 nm of Fpg or hOGG1 (New England Biolabs) was added. For probe screening, Fpg (833 nm) or hOGG1 (658 nm) was added. The fluorescent emission spectrum was measured at various timepoints following addition of enzyme. The sample was maintained at 37 °C throughout.

PAGE analysis of in vitro enzymatic activity: Probes were labeled at the 5'-end with 32P by using T4 polynucleotide kinase (Invitrogen) and [γ-32P]ATP (PerkinElmer), and purified by ethanol precipitation as previously described.227 BSA (100 µg/mL), unlabelled probe (4 µM), labeled probe (0.05 µM), and Fpg or hOGG1 (658 nm) were mixed in Fpg or hOGG1 reaction buffer and incubated at 37 °C. Aliquots (10 µL) were removed at the indicated timepoints. Non-piperidine-treated aliquots were mixed with loading buffer (10 µL; EDTA (25 mM), formamide (95%), Bromophenol Blue (0.1%), xylene cyanol (0.1%)). Samples to be reacted with piperidine were mixed with piperidine/water (1:10, 2 µL) for 5 min at 90 °C and then mixed with the loading buffer. The samples were frozen for up to 24 h before PAGE analysis. Samples (10 µL) were loaded on a 20% denaturing analytical PAGE gel (20% acrylamide/bis solution (19:1); urea (7.5 M), tris borate EDTA buffer) and electrophoresed. The gel was exposed overnight on a phosphor screen (Molecular Dynamics) and imaged on a Typhoon 9410 gel imager (GE Healthcare).

MALDI-TOF MS analysis of enzymatic activity: Samples were prepared as for the fluorescence assays. An aliquot was submitted to the Stanford Peptide and Nucleic Acid Facility for zip-tip clean up and MALDI-TOF analysis. The samples were analyzed in HPAmatrix solution (19:1), urea (7.5 M), tris borate EDTA buffer) and electrophoresed. The gel was exposed overnight on a phosphor screen (Molecular Dynamics) and imaged on a Typhoon 9410 gel imager (GE Healthcare).

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E. coli: One Shot BL21(DE3) cells (Invitrogen/Life Technologies) were transformed with plasmid pKK223.3-WT Fpg (a gift from Prof. Sheila David, University of California–Davis)228 by following the Invitrogen protocol. A single colony was cultured overnight at 30 °C in lysogeny broth (LB) containing ampicillin (50 µg/mL). The culture was diluted in LB containing ampicillin (50 µg/mL) to OD600 = 0.1 and grown at 37 °C to OD600 = 0.4. Isopropyl-1-thio-β-D-galactopyranoside (IPTG, 1 mM) was added, and the culture was grown for 3 or 24 h at 30 °C. The cells were washed in PBS, centrifuged (5000 g, 5 min, 4 °C), and resuspended in Fpg reaction buffer (to OD600 = 0.6). Triton X-100 (0.25%) and probe (4 µM) were added. Samples were incubated with shaking at 37 °C for 1, 4, or 17 h, then diluted (1:10) in water, and fluorescence emission was measured on a Jobin Yvon-Spx Fluorolog 3 spectrophotometer.

Mammalian cell extracts: Ogg1−/− MEF cells (a gift from Dr. Istvan Boldogh, University of Texas Medical Branch at Galveston)229 and WT MEF cells were grown in DMEM/F12 (3:1) (HyClone) supplemented with fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified incubator at 37 °C with 5% CO2. Cell extracts were obtained by the protocol by Folco et al.230 with a few changes: the cells were collected by trypsinization at room temperature instead of scraping; Roche complete EDTA-free tablets were used instead of PMSF; the cells were lysed by passing 10–15 times through a 21 gauge needle and 5–10 times through a 16 gauge needle instead of through a Dounce homogenizer. Total protein concentration was determined by a Bradford assay.

Equal amounts (6.7 ng/µL) of WT and Ogg1−/− MEF cell extract were incubated with probe pOG1 (2 µM) and MgCl2 (10 mM) at 37 °C in 80 µL reaction volumes in a black 384-well plate. Fluorescence was measured at 460 nm on a Fluoroskan Ascent FL fluorescence plate reader (Thermo Scientific; λex = 355 nm).

Melting temperature measurements: Samples containing probe OGR1 (4 µM) in Fpg or hOGG1 reaction buffer reaction buffer were prepared in stoppered 1 cm path-length quartz cells. A Varian Cary 100 UV/Vis spectrophotometer equipped with a thermostepprogramm er was used to measure absorbance (260 nm) while the temperature was changed from 25 to 99 °C and from 99 to 25 °C (1 °C/min). Because of the high melting temperature of OGR1 and the lack of an absorbance plateau in the high temperature range, MeltWin software could not be used to determine the melting temperatures; melting temperatures were determined by visual inspection of the melting curves and by approximation of the first derivative of the melting curve.

Measurement of kinetic parameters: Michaelis–Menten curves for hOGG1 with and without inhibitor (3′-C4′-F-indole-containing oligonucleotides) were generated by preincubating hOGG1 (0.11 µM) with/without inhibitor (2 µM) in hOGG1 reaction buffer with BSA (100 µg/mL) at 37 °C for 5 min. OGR1 (0.5–10 µM) was then added at 37 °C, and the initial velocity was evaluated (rate of fluorescence increase (linear) at 460 nm over the first 10 min).

Michaelis–Menten curves for Fpg were generated by incubating OGR1 (0.03–0.3 µM) with Fpg (6 nm) in Fpg reaction buffer with
BSA (100 µg mL⁻¹) at 37 °C and evaluating the initial velocity at 37 °C (rate of fluorescence increase linear) at 460 nm over the first 6 min.

The curves were fitted in Origin (OriginLab, Northampton, MA) by using the Hill fit with n = 1 (yielding the Michaelis–Menten equation) to determine Kₚ and Vₘₐₓ. The dissociation constant, Kₚ, of the hOGG1 inhibitor was calculated from apparent Kₚₐ: Kₚₐ = Kₒₚₛ /[enzyme].

To calculate kₑₜₐₜ (Kₒₚₛ = Vₘₐₓ/[enzyme]), we made a calibration curve to convert fluorescence units to amount of product by incubating known concentrations of probe with excess Fpg and measuring the fluorescence increase after the fluorescence signal plateaued. Vₘₐₓ was converted from fluorescence units per s to µmol product per s from the calibration curve.

384-well assay: OGR1 (0.8 µM) and hOGG1 (150 nM) were incubated in hOGG1 reaction buffer with BSA (100 µg mL⁻¹) at 37 °C in 20 µL reaction volumes in a black 384-well plate. Fluorescence at 460 nm was measured on a Thermo Fluoroskan Ascent FL fluorescence plate reader (λₑₓ = 355 nm). The signal-to-noise ratio was calculated by dividing the signal of OGR1 with hOGG1 by the signal of OGR1 without hOGG1. The coefficient of variance was calculated of OGR1 without hOGG1. The coefficient of variance was calculated by dividing the standard deviation by the average signal intensity at that time point. Z factor was calculated from:

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1 - \frac{3 \times SD_{signal} + 3 \times SD_{baseline}}{mean_{signal} - mean_{baseline}}
\]

(1)

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