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Naturally occurring polyphenol, morin hydrate, inhibits enzymatic activity of *N*-methylpurine DNA glycosylase, a DNA repair enzyme with various roles in human disease

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Abstract

Interest in the mechanisms of DNA repair pathways, including the base excision repair (BER) pathway specifically, has heightened since these pathways have been shown to modulate important aspects of human disease. Modulation of the expression or activity of a particular BER enzyme, *N*-methylpurine DNA glycosylase (MPG), has been demonstrated to play a role in carcinogenesis and resistance to chemotherapy as well as neurodegenerative diseases, which has intensified the focus on studying MPG-related mechanisms of repair. A specific small molecule inhibitor for MPG activity would be a valuable biochemical tool for understanding these repair mechanisms. By screening several small molecule chemical libraries, we identified a natural polyphenolic compound, morin hydrate, which inhibits MPG activity specifically ($IC_{50} = 2.6 \mu M$). Detailed mechanism analysis showed that morin hydrate inhibited substrate DNA binding of MPG, and eventually the enzymatic activity of MPG. Computational docking studies with an x-ray derived MPG structure as well as comparison studies with other structurally-related flavanoids offer a rationale for the inhibitory activity of morin hydrate observed. The results of this study suggest that the morin hydrate could be an effective tool for studying MPG function and it is possible that morin hydrate and its derivatives could be utilized in future studies focused on the role of MPG in human disease.

Keywords

base excision repair; carcinogenesis; chemosensitization; neurodegeneration; enzyme inhibitors

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1. Introduction

The study of DNA repair is highly significant in human health since DNA repair pathways modulate both carcinogenesis and resistance to chemotherapy as well as neurodegenerative diseases [1–4]. In the area of carcinogenesis, DNA repair has traditionally been understood to be a barrier to cancer, but recent work has shown that overactive DNA repair can be associated with higher risk for cancer. Specifically, overexpression of some DNA repair enzymes, contrary to expectation, confers increased genomic instability and risk for cancer [5, 6]. *N*-methylpurine DNA glycosylase (MPG)⁴, an important initiating glycosylase in the base excision repair (BER) pathway, the major cellular repair pathway for alkylated, oxidized, or deaminated bases, has been shown to be involved in carcinogenesis. In particular, MPG, a DNA glycosylase that excises a variety of alkylated purines, has been shown to induce frameshift mutagenesis and microsatellite instability in yeast as well as in human cells [7]. In humans MPG overexpression is also associated with higher risk for lung cancer [8]. Of note, in patients with ulcerative colitis (UC), a pre-malignant chronic inflammatory condition associated with increased microsatellite instability (MSI) and risk for colorectal cancer, it was found that inflamed tissues displayed increased MPG expression and activity levels [5].

Additionally, MPG has been shown to confer resistance to a chemotherapeutic alkylator, temozolomide (TMZ) in glioblastoma multiforme [9]. Knockdown of MPG in HeLa and ovarian cancer cells also enhanced cytotoxicity of MMS, *N*-methyl-*N*-nitrosourea (MNU), and TMZ [10]. Taken together, these studies indicate that MPG overexpression may play a role in carcinogenesis as well as chemotherapeutic resistance and requires more study.

MPG also has a complex role in neurodegeneration. Deficiency in MPG has been shown to induce alkylation-induced neuronal cell death in culture [11]. Conversely, overexpression of MPG was shown to increase cerebellar toxicity as well as retinal degeneration in mice [12].

The availability of a specific small molecule inhibitor for MPG activity would be beneficial to future work on MPG as a target for chemoprevention, chemosensitization, and neurological disease studies. Several metals have been shown to inhibit DNA glycosylases. MPG is inhibited by Mg^{2+} while NEIL1 is inhibited by both Fe^{2+} and Cu^{2+} [13, 14]. However, these metals may not be useful as repair inhibitors in *in vivo* studies as they may have many non-specific effects.

The goal of the present study was to discover and characterize an inhibitor to be used in the study of basic mechanisms of MPG-directed BER. We first screened compound libraries for small molecules that inhibited the enzymatic activity of MPG and did not bind to DNA. Interestingly, a naturally occurring polyphenolic compound morin hydrate emerged from the screens as an MPG inhibitor.

⁴The abbreviations used are *N*-methylpurine DNA glycosylase (MPG), base excision repair (BER), apurinic/aprimidinic endonuclease 1 (APE1), 8-oxo-guanine DNA glycosylase (OGG1), 1, *N*⁶-ethenoadenine (ϵ A)

2. Materials and Methods

2.1 Compound libraries

The Sigma-Aldrich Library of Pharmacologically Active Compounds (LOPAC¹²⁸⁰) and the Natural Product Screening Library (NPSL) from SelleckChem were received as 10 mM DMSO stock solutions and used as described below in the gel-based excision activity assay for MPG.

2.2 Virtual library screening

For virtual inhibitor screening, three *in silico* strategies were implemented: (a) generation of flexible protein conformations of MPG to mimic the dynamicity of the protein, (b) small molecular docking simulations, and (c) post-docking re-ranking of compounds using in-house developed “train, match, fit, streamline” (TMFS) method [15]. For flexible protein conformations, nano-second molecular dynamic simulations were performed. Conformations were then clustered and a representative conformation in addition to x-ray conformation was selected for small molecule screening [16]. Docking simulations were carried out over $\approx 250,000$ pre-selected (target specific) commercially available compounds over in-house chemical library. A narrow window of 2,500 commercial compounds was selected based on ranking with an arbitrary energy cut-off. Final ranking of compounds was done using the TMFS method, and the top 57 compounds were selected for further testing by the gel-based activity assay.

2.3 Gel-based excision activity assay

Purified hMPG (2.3 nM) was pre-incubated with 20 μ M of each compound for 10 min at room temperature. The pre-incubated mixes were subsequently incubated with 7 nM 1, N⁶ ethenoadenine (ϵ A)-containing ³²P-labeled duplex oligonucleotide substrates (5'-TCGAGGATCCTGAGCTCGAGTTCGACGXTTCGCGAATTCTGCGGATCCAAGC-3'), where X = ϵ A, for 10 mins at 37°C in an assay buffer containing 25 mM HEPES, pH 7.9, 150 mM NaCl, 100 μ g/mL BSA, 0.5 mM DTT, and 10% glycerol in a total volume of 20 μ L. The MPG reactions were terminated at 65°C for 10 min then cooled to room temperature for 15 min. AP-sites were cleaved with a reaction mixture of 15 nM apurinic/aprimidinic endonuclease 1 (APE1) and 5 mM MgCl₂ at 37°C for 10 min. Reactions were diluted 1:1 with a loading buffer containing 1X gel loading dye (New England Biolabs, Ipswich, MA) and 85% formamide. Samples were subsequently heated at 95°C for 3 min followed by cooling on ice for 3 min. Samples were resolved by denaturing gel electrophoresis at 60°C using Criterion gel cassettes (BioRad, Hercules, CA) containing 20% polyacrylamide (BioRad, Hercules, CA) and 7M urea (BioRad, Hercules, CA). Radioactivity was quantified by exposing the gel to X-ray films and quantifying the band intensities using an imager (Chemigenius Bioimaging System, Frederick, MD) and software (GeneTool, Syngene Inc., San Diego, CA). Reactions to test 8-oxoguanine DNA glycosylase (OGG1) and APE1 activity were performed similarly, using appropriate radiolabeled duplex substrate oligonucleotides. Both OGG1 and APE1 were cloned, expressed, and purified previously [17]. Oligonucleotides containing 8-oxo-dG were used for OGG1 activity assays, which were performed in the same buffer system utilized in the MPG activity assay.

Oligonucleotides containing tetrahydrofuran (THF), a stable AP-site analog, were used for APE1 activity assays in which the assay buffer was supplemented with 5 mM MgCl₂.

Reactions using whole cell extracts were performed similarly, using 5 µg A549 or HeLa extract (prepared using M-PER buffer according to manufacturer's protocol; Sigma-Aldrich) and 3 nM εA-containing ³²P-labeled oligonucleotide substrates. Extracts were similarly pre-incubated with increasing doses of morin hydrate or quercetin (0, 50, 100, 200, and 300 µM) for 10 min at room temperature before incubation with substrate oligonucleotides.

2.4 Surface plasmon resonance studies

Binding studies were performed in a Biacore T100 system (Biacore, Uppsala, Sweden) as described previously with some modifications [18]. To test the affinity of selected compounds for DNA, a 50-mer oligonucleotide containing εA or an undamaged oligonucleotide (same sequence as described for the gel-based activity assay) were biotinylated and immobilized on streptavidin-coated C1 Biacore chips. Then RU values were recorded with three injections of mitoxantrone dihydrochloride, gossypol, or morin hydrate (15 µM) in a binding buffer containing 10 mM HEPES-KOH, pH 7.6, 90 mM KCl, and 0.05% surfactant P20 (Biacore, Uppsala, Sweden) at 7°C. To study inhibition of hMPG binding to substrate DNA in the presence of morin hydrate, the εA-containing oligonucleotide was immobilized and RU values were recorded with injections of hMPG pre-incubated with increasing concentrations of morin hydrate (0, 5, 10, 20, 40 µM).

2.5 Molecular docking studies

Docking of Morin Hydrate to the crystal structure of the hMPG complexed with εA-containing DNA (PDB-ID 1EWN) was performed using Autodock Vina 1.1 in the flexible docking mode to the energy minimized structure (<http://vina.scripps.edu/index.html>). The grid box parameters for autodock runs were initially generated to cover the entire surface of the molecule. After closer inspection only one site was evident as a binding site and the grid box was adjusted accordingly. The protein residues at hydrogen bonding distance to morin hydrate was determined using PDBSum (<https://www.ebi.ac.uk/pdbsum/>).

2.6 Cell culture

A549 and HeLa cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic.

2.7 In vitro construct preparation

The preparation of the control, εA-, and 8-oxo-dG-containing plasmid DNA was performed *in vitro* as previously described with some modification [19]. The lesion (εA or 8-oxo-dG) was placed at the EcoRI site of the M13mp18 phagemid. Typically, for each preparation of M13mp18-ctrl, -εA, or -8-oxo-dG plasmid, 6 individual reactions were performed simultaneously according to the following protocol. Phosphorylation of the primers was performed by incubating 2 µg of εA - containing (5'-CCGAGCTCGXATTCGTAATC-3'), where X = εA or 8-oxo-dG-containing (5'-CCGAGCTCXAATTCGTAATC-3'), where X = 8-oxo-dG, oligonucleotide (2 µL) with 1X PNK buffer, 400 nM ATP, 50 mM DTT, and 10 U of T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) in a 30 µL reaction

volume at 37°C for 45 mins. The phosphorylated oligonucleotide was purified through a G-25 column (GE Healthcare Life Sciences, Pittsburgh, PA) according to the manufacturer's protocol. Then 6 µL of this purified oligonucleotide was incubated with 2 µg of M13mp18 ssDNA in an annealing buffer containing 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl in a 20 µL reaction volume. This annealing reaction was incubated at 80°C for 5 minutes and slowly cooled to room temperature with brief centrifugation when the reaction reached 50°C. Then the annealing reaction was incubated with an extension reaction mixture containing 1X T7 DNA polymerase buffer, 1.5 mM ATP, 1.5 mM of each dNTP, 10 mM DTT, and 160 µg/mL BSA, 10 U of T7 DNA polymerase (New England Biolabs, Ipswich, MA), and 400 U of T4 DNA ligase (New England Biolabs, Ipswich, MA) in a final reaction volume of 30 µL for 5 mins on ice followed by 5 mins at room temperature. The extension reaction was subsequently incubated at 37°C for 1 h. After an hour, 50 nmol of ATP and 200 U of T4 DNA ligase were added to the extension reaction and incubated at 14°C overnight for efficient ligation to occur. The 6 individual reactions were then pooled together and incubated with 1X Supercoil-It buffer (Bayou Biolabs, Metairie, LA) and 2 µL of Supercoil-It enzyme mixture at 37°C for 3 h. Plasmid DNA was recovered after the incubation by purification using Qiaquick PCR Purification kit (Qiagen, Gaithersburg, MD). The DNA was eluted from the column using 50 µL of molecular grade water. Concentration of the eluted DNA was measured using a Nanodrop spectrophotometer, and the DNA was stored at -20°C for use in the *in vivo* repair assays.

2.8 In-cell repair assay

The in-cell repair assay was performed as previously described [19]. Briefly, post-transfection, the cells were harvested and the plasmids were recovered using Qiagen Miniprep kit (Qiagen). Retrieved plasmid DNA was aliquoted and digested individually with EcoRI to (εA or 8-oxo dG site) and SacI (internal control site). Digested plasmid DNA was used for plaque formation and repair calculations were performed, both as described previously [19].

2.9 MTT assay

Cells (A549 and HeLa) were seeded in a 96-well plate (2000 cells per well). The following day cells were treated for 72 h with increasing doses of morin hydrate in triplicate (0, 0.1, 0.2, 0.4, 0.8, 1, 2 mM). Following incubation the media was aspirated, and 100 µL of a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3,4-diphenyl)-tetrazolium bromide (MTT) (Sigma-Aldrich) solution (0.5 mg/mL in DMEM) was added to the cells and incubated for 4 h at 37°C. The MTT solution was subsequently aspirated and cells were incubated for 30 min shaking at room temperature with 100 µL DMSO for solubilization. Absorbance was measured at 570 nm and 650 nm (background). Absorbance values at 570 nm were background subtracted, and percent survival was calculated according to the untreated control wells.

3. Results

3.1 Inhibitor screening

Overall, the goal of small molecule screening was to find compounds that inhibited MPG excision activity without themselves binding to DNA in order to ensure better specificity of inhibition. To find such an inhibitor of MPG, three unique libraries of small molecules were screened for inhibition of MPG excision activity using a gel-based assay with ϵ A-containing duplex oligonucleotides as substrate for MPG (see Materials and Methods) (Figure 1). Ethenoadenine was used as an MPG substrate throughout the study since the crystal structure of MPG bound to ϵ A is available, which facilitated the molecular docking experiments. Compounds that inhibited >80% of MPG excision activity were considered for further testing for DNA binding inhibition and specificity. The first was a library of potential inhibitors that were designated as such based on virtual structural analysis (Figure 1A). Another library screened for potential inhibitors was the LOPAC¹²⁸⁰ pharmacologically active compound library commercially available from Sigma-Aldrich (Figure 1B). Finally, a natural compound library available from SelleckChem was screened for possible MPG inhibitors (Figure 1C). Among the 3 libraries screened, 6 candidate inhibitors were selected for further testing: two arbitrarily named compounds from the virtual library, aurintricarboxylic acid (ATA) and mitoxantrone dihydrochloride (from LOPAC¹²⁸⁰), and gossypol and morin hydrate (from NPSL).

After further testing, the two compounds chosen from the virtual library were eliminated from further consideration since both were found to be promiscuous inhibitors of MPG. The IC₅₀ of inhibition and structure for each of the remaining compounds is listed in Table 1. The IC₅₀ for each compound was determined using a range of concentrations of the inhibitor in the gel-based assay. A representative gel image and quantification is shown for morin hydrate in Figure 2A and 2B. Of note, ATA was also eliminated from further consideration as an MPG inhibitor as it is a known disruptor of many protein-nucleic acid interactions [20]. ATA seems to disrupt these interactions both by binding DNA and by binding proteins [20–22] therefore it was not considered for further testing.

The ideal MPG inhibitor should inhibit the binding of MPG to DNA but not bind to DNA itself, which could potentially result in a lack of inhibitor specificity. Therefore, the remaining three compounds, morin hydrate, gossypol, and mitoxantrone dihydrochloride, were tested for DNA binding affinity using SPR technology. While both gossypol and mitoxantrone dihydrochloride demonstrated affinity for DNA, no detectable affinity for unmodified (control) or ϵ A-containing DNA could be observed for morin hydrate (Figure 3A and 3B). Of note, the MTX results were consistent with the literature as MTX is also a known DNA intercalator [23]. This is presumably its mechanism of MPG inhibition, which was unsuitable for our purposes due to predicted non-specific inhibition of many DNA-protein interactions. Gossypol has been demonstrated to damage DNA through free radical formation [24], and some evidence has been shown that gossypol can be oxidized by Cu²⁺ to form a gossypol radical that can directly bind DNA [25]. Our SPR study, which did not include Cu²⁺, demonstrated that gossypol itself has some affinity for DNA (Figure 3A and 3B). As was the case for MTX, the affinity of gossypol for DNA may have been then

mechanism of MPG inhibition in the gel-based activity assay. Morin hydrate was the only compound that did not demonstrate any measurable affinity for either unmodified DNA or DNA with MPG substrate lesions (Figure 3A and 3B). Again, utilizing SPR technology, increasing concentrations of morin hydrate were incubated with MPG in the presence of immobilized ϵ A-containing oligonucleotides, resulting in a dramatic decrease in affinity of the enzyme for substrate DNA (Figure 3C). The IC_{50} of binding inhibition was 12 μ M (Figure 3D), which was consistent with the IC_{50} of activity inhibition determined by the gel-based assay (Figure 2B). Complete inhibition of DNA binding occurred in the presence of 40 μ M morin hydrate (Figure 3C and 3D). Morin hydrate inhibited the activity of MPG via disruption of DNA binding, revealing it to be a potentially useful and relevant MPG inhibitor for MPG-related studies.

3.2 Morin hydrate specificity

The ideal MPG inhibitor should be specific, so the effect of morin hydrate on two other known BER enzymes, APE1 and OGG1, was tested (Figure 4). APE1 was used (in molar excess) in the gel-based activity assay for MPG, so it was important to determine the effect of the compound on APE1. OGG1 is a DNA glycosylase and could potentially be inhibited by morin hydrate via a similar mechanism as MPG. However, morin hydrate demonstrated no effect on the activity of APE1 as depicted in the representative gel image and quantification in Figures 4A and 4B. Interestingly, some promiscuous inhibition of OGG1 was observed (Figures 4C and 4D), but this was not deemed significant since the effect was not dose-dependent. Of note, morin hydrate was shown to inhibit some other BER proteins, including both DNA polymerase β and APE1, in high throughput screening assays (Pubchem Bioassay AID 485314 and 2517) although our gel-based assay did not confirm those results for APE1.

3.3 Molecular modeling and preliminary proof of modeling-based structure-activity studies

To better understand the binding interactions between morin hydrate and MPG, a structural model of the morin hydrate/ MPG complex was developed. Molecule docking of the compound morin hydrate into the ligand binding model site of MPG was performed on the binding model based on the MPG- ϵ A complex structure (PDB: 1EWN). Closer examination of the structure of morin hydrate and the predicted model of its interaction with MPG are shown in Figure 5B. Virtual modeling demonstrates that morin hydrate interacts with key amino acid residues in the DNA binding domain of MPG: His136, Ala134, and Val262 [26] (Figure 5C). These interactions are at the interface between MPG and DNA, which may be the source of morin hydrate's inhibition of MPG (Figures 5D). Specifically, the functional groups on the inhibitor that interact with these residues are the hydroxyl (OH) groups characteristic of flavonoids [27] (Figure 5E). The 2' OH found on the B ring of morin hydrate (Figure 5A) putatively interacts with Val262 (Figure 5E) while the 5' OH on the A ring (Figure 5A) may interact with both Ala134 and His136 (Figure 5E). Of note, His136 directly interacts with DNA during substrate binding [26].

To determine the key substituted OH groups involved in MPG inhibition, a variety of naturally occurring flavonoid compounds were tested for inhibition using the gel-based activity assay. The flavonoids tested and the observed percent inhibition of MPG activity are

listed in Table 2. Quercetin, myricetin, and kaempferol are 3,5,7-OH substituted (on the A and C rings) flavonoids like morin hydrate. However, the OH pattern on the B ring in those compounds is slightly different. Of note, quercetin contains both a 3' and 4'-OH on the B ring while morin hydrate contains a 2' and 4'-OH on the B ring. Only 44% MPG inhibition was observed in the presence of quercetin while 81% inhibition was observed in the presence of morin hydrate, suggesting that the 2'-OH on the B ring is a critical functional group responsible for the strong inhibitory effect of morin hydrate on MPG. Interestingly, a comprehensive study of the binding and active sites of MPG in complex with ϵ A-containing DNA demonstrated that while His136 and Ala134 are involved in substrate binding, Val262 is involved in catalysis [26]. Then the results of the structure-activity relationship study with morin hydrate and quercetin suggest that quercetin and other 3,5-OH substituted flavonoids may also inhibit the binding of MPG to DNA. However, morin hydrate with its additional 2'-OH on the B ring exhibits more effective inhibition of the enzyme.

3.4 Morin hydrate inhibition of MPG in cell extracts

Increasing concentrations of morin hydrate were tested for their effect on MPG in human whole cell extracts (Figure 6). A549 cell (lung adenocarcinoma) and HeLa cell (cervical cancer) extracts were tested since they have been used for other MPG-related studies in other laboratories [10, 28]. MPG activity in both A549 and HeLa whole cell extracts was inhibited in the presence of morin hydrate in a dose-dependent manner (Figure 6A). However, it should be noted that the estimated IC_{50} of inhibition in cell extracts was higher with extracts (average between two cell lines ≈ 100 – $200 \mu M$; Figure 6A) than with purified enzyme ($2.6 \mu M$; Figure 2). Quercetin, did not have an effect on MPG activity in either of the cell extracts, correlating with the observed results using purified MPG (Figure 6B and Table 2).

3.5 Morin hydrate inhibition of MPG in live cells

In addition to morin hydrate's ability to inhibit MPG *in vitro* and in cell extracts, we also tested its ability to enter human cells and exert its effect *in vivo*. Ethenoadenine-containing and 8-oxodG-containing M13mp18 plasmids were assayed for complete repair in A549 and HeLa cells in the presence of increasing concentrations of morin hydrate using our previously described plaque assay method [19] (Figure 7). In both cell lines, repair of eA *in vivo* was inhibited by morin hydrate in a dose dependent manner while repair of 8-oxo-dG *in vivo* was unaffected by treatment with the compound (Figure 7A). Interestingly, 50–60% reduction in repair was observed at $300 \mu M$ morin hydrate, which is consistent with the IC_{50} observed in cell extract experiments (Figure 6A). Notably, the concentrations of morin hydrate used on the A549 and HeLa cells were not toxic (Figure 7B). Repair of 8-oxo-dG served as a control experiment in two ways. First, *in vitro* experiments with purified enzymes indicated that morin hydrate exerted promiscuous inhibition of the excision activity of OGG1, so monitoring repair of 8-oxo-dG can demonstrate specificity of the inhibitor *in vivo* (Figure 4C and 4D). Since we did not observe inhibition of 8-oxo-dG repair in live cells treated with morin hydrate, we concluded that the compound does not have any appreciable effect on OGG1 activity. Secondly, morin hydrate has been shown to act as an antioxidant, so it is possible that the compound could inhibit MPG *in vivo* via alterations in redox status in the cells even though no evidence of MPG redox-sensitivity has been reported. OGG1, a

redox sensitive enzyme, demonstrated no alterations in repair activity *in vivo* under morin hydrate treatment while MPG activity was inhibited, indicating that the compound most likely did not inhibit MPG via its antioxidant properties (Figure 7) [29].

4. Discussion

The present study reports the screening of 3 small molecule libraries for potential inhibitors of a DNA repair protein, MPG. The first round of screening utilized a gel-based MPG activity assay as the read-out for inhibition. Out of that study, several potential inhibitors were selected, but after screening the selected compounds for DNA binding affinity, one compound, morin hydrate, was selected for further analysis. The results of this study demonstrate that morin hydrate, a natural compound, inhibits the DNA binding and activity of MPG (Figure 2, 3C, and 3D). Of note, it does not bind to DNA itself, which is an important characteristic to ensure inhibitor specificity (Figure 3A and 3B). Moreover, morin hydrate does not inhibit OGG1 and APE1, two prominent BER enzymes, indicating that it is a specific inhibitor of MPG (Figure 4). The results of the present study suggest that morin hydrate could be an effective tool for studying MPG function and could potentially be utilized in studies focused on the role of MPG in various aspects of human disease.

Since this study has shown that morin hydrate inhibits MPG at a relatively low concentration ($IC_{50} = 2.6 \mu M$; Figure 2) *in vitro*, there is a useful opportunity to utilize this inhibitor in studies of the basic mechanism of MPG and BER in general. This includes a variety of *in vitro* experiments, including those focused on the mechanism of adduct excision as well as reconstitution studies that include the other members of the BER pathway. Additionally, our comparison of morin hydrate with other related flavanoid compounds provides valuable insight about the crucial –OH groups involved in the inhibition of MPG (Table 2) and lays the groundwork for future derivative development for more potent inhibition.

MPG is an important biological enzyme, so it should be noted that the IC_{50} of MPG inhibition by morin hydrate in live cells and in extracts was higher (100–300 μM ; Figures 6–7) than the IC_{50} of inhibition of purified recombinant MPG (2.6 μM ; Figure 2). Importantly, inhibition was still specific even at high concentrations (Figure 7). Therefore, to inhibit MPG for biological applications, higher concentrations of morin hydrate should be used. It is known that polyphenols interact with metal ions, which can change a polyphenol's chemical reactivity toward cellular components [25]. One concern may be that the presence of metal ions in the live cell repair assay somehow abrogated the potency of morin hydrate against MPG even though under these conditions, morin hydrate inhibited the activity of MPG. The results indicated that the presence of metal ions did not completely disrupt the interaction between MPG and morin hydrate (Figure 7A). Nonetheless, the IC_{50} of inhibition was much higher in live cells than in the *in vitro* experiments with purified protein. However, the IC_{50} of inhibition in morin hydrate-treated cell extracts was similar to that in live cells. There is certainly a decrease in metal ion concentration in cell extracts due to chelating agents present in the lysis buffer, so we hypothesize that the increase in IC_{50} was probably due to binding non-specifically to other cellular proteins. This highlights the

importance of our future goal, which is to identify derivatives of morin hydrate that may be more potent inhibitors of MPG, particularly in the context of live cells and extracts.

We noted earlier that MPG is a significant molecule in both carcinogenesis, chemoresistance, and neurodegenerative conditions. Therefore, it is tempting to consider the role that morin hydrate may play in mediating many of these aspects of human disease by inhibiting MPG. In some colon and tongue carcinogenesis models in rats, morin hydrate, via its anti-oxidant and anti-inflammatory characteristics, exerted a chemopreventive effect [32–34]. Also, many studies have demonstrated that polyphenols sensitize cancer cells to chemotherapy via effects on survival and growth signaling pathways [35, 36]. Moreover, morin hydrate has been shown to exert a neuroprotective effect via its antioxidant properties in mouse models of Parkinson's disease [37] and against excitotoxic neuronal death in culture [38]. However, the relatively high IC₅₀ in live cells and in extracts would not be conducive to using morin hydrate to inhibit MPG-induced carcinogenesis, resistance to chemotherapy, or alkylation damage-induced neurotoxicity. It is, however, intriguing to consider that a more potent derivative of morin hydrate, once developed, might be a useful small molecule in the aforementioned health issues. Importantly, this study provides a solid foundation for the pursuit of a morin hydrate derivative for such purposes.

5. Conclusions

Through screening several small molecule chemical libraries we identified a natural polyphenolic compound, morin hydrate, which specifically inhibits MPG activity. Detailed mechanism analysis showed that morin hydrate inhibited substrate DNA binding of MPG, and eventually the enzymatic activity of MPG. Computational docking studies with an x-ray derived MPG structure offer a rationale for the inhibitory activity of morin hydrate observed. This computational model as well as our comparison studies with other flavanoid compounds may facilitate development of morin hydrate derivatives with improved MPG inhibiting activity. In summary, the results of this study suggest that the morin hydrate could be an effective tool for studying MPG function. Investigations of MPG function are particularly important since MPG plays an important role in a variety of human diseases, and morin hydrate and its derivatives may be useful in studies elucidating the effect of MPG in those diseases.

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Figure 1. Small molecule library screening

MPG activity was tested in the presence of small molecules by the gel-based activity assay. Graphs indicate the categorization of compounds by level of MPG activity inhibition. (A) Virtual library (B) LOPAC¹²⁸⁰ (C) NPSL.

Figure 2. Morin hydrate inhibits MPG activity

(A) Representative image from gel-based activity assay for MPG in the presence of increasing doses of morin hydrate (0–8 μM). (B) Quantification of inhibition of MPG activity by morin hydrate.

Figure 3. Morin hydrate does not bind to DNA and inhibits MPG binding to substrate DNA
 The binding of mitoxantrone dihydrochloride, gossypol, and morin hydrate to undamaged (control) (A) and ϵ A-containing (B) oligonucleotides was measured by SPR. Sensorgram depicts the background-subtracted RU values for two 60 s injections for each compound. (C) MPG binding to ϵ A-containing oligonucleotides in the presence of increasing concentrations of morin hydrate was measured by SPR. Sensorgram depicts the background-subtracted RU values for two 60 s injections of 30 nM MPG pre-mixed with each concentration of morin hydrate. (D) Graph indicates the percent inhibition of MPG binding in the presence of increasing doses of morin hydrate (0–40 μ M) determined from SPR experiment. Inhibition is calculated as the percent decrease in background-subtracted RU values for MPG binding in the presence of morin hydrate compared to DMSO.

Figure 4. Morin hydrate does not inhibit other key BER enzymes

Gel-based activity assays were performed for both APE1 and OGG1 in the presence of increasing doses of morin hydrate (0, 20, and 40 μM). (A) Representative image of APE1 activity assay with morin hydrate. (B) Quantification of APE1 activity assay (C) Representative image of OGG1 activity assay with morin hydrate. (D) Quantification of OGG1 activity assay.

Figure 5. Molecular docking studies with MPG and morin hydrate

(A) Chemical structure of morin hydrate. The three rings of the flavonoid backbone are labeled, A, B, and C, and the positions of the –OH groups are denoted. (B) Morin hydrate was modeled in the crystal structure of MPG (PDB: 1EWN) in absence of DNA, (C) Representation of the interaction between morin hydrate and select MPG amino acids. (D) Morin hydrate was docked in MPG-DNA complex (PDB: 1EWN). The green sticks represent morin hydrate and the backbone of DNA shown as orange cartoon, (E) Hydrogen

bonding (shown as green dotted lines) and van der waals interactions (shown in red in the shape of a half-moon) between the docked morin hydrate and MPG.

A **B** **Figure 6. Morin hydrate inhibits MPG activity in cell extracts**

Increasing doses of morin hydrate were incubated with 5 μg of whole cell extract from A549 and HeLa cells and evaluated by the gel-based assay. (A) Representative images of MPG activity assay in whole cell extracts with morin hydrate. (B) Representative images of MPG activity assay in whole cell extracts with quercetin. Graphs indicate the quantification of the percent product formation in the gel-based assays, normalizing to untreated samples.

Figure 7. Morin hydrate specifically inhibits MPG activity *in vivo*

(A) Repair of ϵ A and 8-oxo-dG in A549 and HeLa cells in the presence of increasing doses of morin hydrate (0, 200, and 300 μ M) as determined by the plasmid-based in-cell repair assay (see Materials and Methods). (B) Survival of A549 and HeLa cells in the presence of increasing doses of morin hydrate.

Table 1

Screening hits for MPG.

Library Screened	Compound	IC ₅₀ (μM) ^a	Chemical Structure
LOPAC 1280	aurintricarboxylic acid (ATA)	0.2	
LOPAC 1280	mitoxantrone dihydrochloride	2.5	
NPSL (130)	gossypol	3.0	
NPSL (130)	morin hydrate	2.6	

^aIC₅₀ determined by gel-based activity assay

Table 2

Structure-activity relationship studies

Compound	Structure ^b	MPG Inhibition ^a
Morin hydrate		81%
Quercetin		44%
Quercetin dihydrate		38%
Myricetin		36%
Kaempferol		25%
Caffeic acid		0%
Cinnamaldehyde		0%

^aPercent inhibition determined using gel-based activity assay with 20 μ M compound.^bBlue letters are the flavanoid ring indicators. Red numbers indicate the positions of the substituted -OH groups.