The selectivity and inhibition of AlkB


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Summary

AlkB is one of four proteins involved in the adaptive response to DNA alkylation damage in *Escherichia coli* and is highly conserved from bacteria to humans. Recent analyses have verified the prediction that AlkB is a member of the iron (II) and 2-oxoglutarate (2OG) dependent oxygenase family of enzymes. AlkB mediates repair of methylated DNA by direct demethylation of 1-methyladenine and 3-methylcytosine lesions. Other members of the iron (II) and 2OG dependent oxygenase family, including those involved in the hypoxic response, are targets for therapeutic intervention. Assays measuring 2OG turnover were used to investigate the selectivity of AlkB. 1-Methyladenosine, 1-methyl-2′-deoxyadenosine, 3-methylcytidine and 3-methyl-2′-deoxycytidine all stimulated 2OG turnover by AlkB, but were not demethylated indicating an uncoupling of 2OG and prime substrate oxidation and that oligomeric DNA is required for hydroxylation and subsequent demethylation. In contrast the equivalent unmethylated nucleosides did not stimulate 2OG turnover indicating that the presence of a methyl group in the substrate is important in initiating oxidation of 2OG. Stimulation of 2OG turnover by 1-methyladenosine was highly dependent on the presence of a reducing agent, ascorbate or dithiothreitol. Following the observation that AlkB is inhibited by high concentrations of 2OG, analogues of 2OG, including 2-mercaptoglutarate, were found to specifically inhibit AlkB. The flavonoid quercetin inhibits both AlkB and the 2OG oxygenase factor inhibiting hypoxia-inducible factor (FIH) *in vitro*. FIH inhibition by quercetin occurs in the presence of excess iron indicating a specific interaction, while the inhibition of AlkB by quercetin is, predominantly, due to non-specific iron chelation.
**Introduction**

The integrity of the genome is maintained by a set of DNA repair enzymes, including those that repair alkylation damage (1). DNA can be alkylated by a variety of agents that occur both exogenously and endogenously (2,3). Alkylating agents are used in some chemotherapy treatments and alkylated DNA bases have been detected in the urine of smokers (4). AlkB catalyses demethylation of DNA and along with AlkA, AidB and Ada is one of four proteins involved in the adaptive response to alkylation damaged DNA in *Escherichia coli* (5). Ada, which has been termed the ‘suicidal repair protein’, contains two active sites, one of which demethylates O\(^6\)-methylguanine by irreversible transfer of the methyl group to a cysteine (5,6). The second active site removes methyl groups from S-methylphosphoesters, by nucleophilic methylation of another cysteine residue (5,7,8). Methylation of the latter cysteine converts Ada into a strong transcriptional activator of both its own production and the other three proteins of the adaptive response (9). AlkA is a DNA glycosylase with a wide substrate selectivity, including excision of cytotoxic 3-methyladenine residues (10). AlkA ‘flips’ the damaged base out of the DNA double helix by inserting Leu-125 into the position occupied by the damaged base (11). The glycosidic bond is then cleaved and the abasic site repaired by the standard base excision repair pathway. AidB is poorly characterised, but it may be involved in the destruction of certain alkylating agents (5).

AlkB is highly conserved from bacteria to mammals (12). *AlkB* knockout mutants are defective at repairing DNA methylation damage induced by S\(_2\) type methylating agents including dimethylsulphate, methyl iodide and methylmethane sulphonate (MMS) (13-15). Recently, two groups have independently confirmed the predictions based upon sequence analyses (16) that AlkB is a member of the non-haem Fe\(^{II}\)
family of oxygenases, requiring a 2-oxoglutarate (2OG) molecule as a cosubstrate (17,18). It was demonstrated that AlkB repairs the cytotoxic 1-methyladenine and 3-methylcytosine lesions in both single and double stranded DNA by an oxidative process (Fig. 1). The reaction most probably proceeds by hydroxylation of the methyl group, leading to its extrusion as formaldehyde. Such a direct repair mechanism is likely to be efficient, as unlike the nucleotide excision repair and base excision repair pathways, it is not reliant on a complementary strand for maintenance of genetic information.

The 2OG dependent family of oxygenase enzymes are involved in a range of metabolic processes in humans including the biosynthesis of collagen and carnitine (19). They also play a key role in the hypoxic response, where it has been proposed that enzymes catalyzing hydroxylations of the hypoxia-inducible factor (HIF) act as oxygen sensors (20,21). Inhibition of the HIF hydroxylases offers potential to develop pro-angiogenic agents, however inhibitors of the asparagine and prolyl hydroxylases will need to be selective for the target 2OG oxygenases.

Here we report work that supports the identification of AlkB as a 2OG dependent oxygenase and demonstrate its inhibition by 2OG analogues. Further, we report that in the presence of the nucleoside substrates 1-methyladenosine, 1-methyl-2′-deoxyadenosine, 3-methylcytidine and 3-methyl-2′-deoxycytidine, there is a stimulation of 2OG turnover by AlkB. However, the 2OG reaction does not appear to be coupled to hydroxylation of the prime substrate.
2. Materials and methods

*Materials* - All chemicals were purchased from Sigma-Aldrich except: oligonucleotides (Sigma-Genosys), restriction enzymes (New England Biolabs), *Pfu* Turbo, ligation kit and competent cells (Stratagene), pET-24a(+) vector (Novagen), Gentra systems kit for purification of *E.coli* DNA, 1-{\[^{14}C\]}-2-oxoglutarate (Perkin Elmer Life Sciences), hyamine hydroxide (ICN radiochemicals) and Opti-phase ‘SAFE’ scintillant (Fisher).

*Cloning* - *AlkB* was cloned from genomic *E. coli* DNA by PCR using the primers; forward 5′-GGTGGTCATATGTTGGATCTGTTTGCC-3′ and reverse 5′-GGTGGTGATCTTCTTTTTTTTACGTG-3′. The PCR product was digested with *Nde* I and *Bam* H I and cloned into pET-24a(+).

*Cell growth* - The pET-24a(+)-*alkB* plasmid was transformed into *E. coli* BL21(DE3) Gold competent cells and growth conditions were optimised. Initially cultures were grown at 37°C until the OD_{600} reached 0.6, where isopropyl-\(\beta\)-D-thiogalactoside was added to a final concentration of 0.2mM and the temperature was dropped to 28°C. Cells were harvested after 4 hours by centrifugation. *AlkB* was produced as ca. 10% of the total soluble protein (by SDS-PAGE analysis).

*Cell lysis and protein purification* - Cells (15g) were resuspended on ice in 50mL of 100mM MES, pH5.8, 1mM dithiothreitol, 40\(\mu\)g mL\(^{-1}\) lysozyme, 4\(\mu\)g mL\(^{-1}\) DNasel, then sonicated (Ultrasonics Inc, W-380) with 4x30s pulses and centrifuged (Beckmann JA25-50) for 20mins at 19000rpm. The supernatant was filtered through a 0.2\(\mu\)m membrane and loaded on to a (50mL) S-Sepharose column (Pharmacia), run at 10mL min\(^{-1}\) in 100mM MES, pH5.8. *AlkB* was eluted with a gradient of 0-1M NaCl over 400mL. *AlkB* containing fractions were pooled and diluted 3 fold prior to loading on a (6mL) Resource-S column (Pharmacia) run at 8mL min\(^{-1}\), again *AlkB*
was eluted with a gradient of 0-1M NaCl over 180mL. AlkB containing fractions were concentrated and loaded onto a (720mL, Superdex-75) gel filtration column at 3mL min\(^{-1}\) in 150mM TRIS, pH7.5. This yielded approximately 30mg of AlkB >95% purity (by SDS-PAGE analysis).

*Methylation of oligonucleotides with MMS* - Methylated DNA oligonucleotides were prepared using a modified version of literature procedures (22,23). Polydeoxyadenine (poly(dA)) and polydeoxycytosine (poly(dC)) were both 15mers. The 27mer and 33mer were of random sequence and contained all four DNA bases. Prior to reaction with MMS oligomers were dissolved at 0.05mM in 10mM TRIS, pH7.5. MMS (240mM in EtOH) was added to a final concentration of 24mM. Reactions were carried out at room temperature for 24 hours. The DNA was precipitated by addition of 3 volumes of ice cold EtOH (0.3M acetate, pH5.2) and placing at -80°C for 30mins. The sample was then centrifuged and the supernatant removed, the precipitated DNA was washed 6 times with EtOH. DNA concentration was measured by UV spectroscopy.

*Methylation of 2′-deoxyadenosine* – 1-Methyl-2′-deoxyadenosine was prepared using the procedure of Singer *et al.* (24). 1-Methyl-2′-deoxyadenosine was the major product, produced in approximately 20% yield (~75% of starting material remained). Yield was estimated by integration of the HPLC trace at 260nm. 1-Methyl-2′-deoxyadenosine was purified by HPLC using a Phenomenex LUNA C-18 column (250×10mm, 5µm). All solvents were filtered through 0.2µm filters and sparged with He (g) at 100mL min\(^{-1}\) for 20mins before use. The column was run at 3.2mL min\(^{-1}\) in 50mM ammonium acetate pH6.4, 5% acetonitrile for 25mins, then a gradient was run to 30% acetonitrile over 5mins and held there for 10mins. Isolated 1-methyl-2′-deoxyadenosine \(m/z\) (ESI+): 266; UV: \(\lambda_{\text{max}}\) (pH7) 259nm, \(\lambda_{\text{max}}\) (pH1) 259nm,
\( \lambda_{\text{max}}(\text{pH13}) \) 260nm, \( \lambda_{\text{inflection}}(\text{pH13}) \) 279nm. As expected these UV profiles were the same as those obtained for the commercial 1-methyladenosine.

**Methylation of 2′-deoxycytidine** – 3-Methyl-2′-deoxycytidine was prepared using the same procedure as for methylation of 2′-deoxyadenosine. Isolated 3-methyl-2′-deoxycytidine \( m/z(\text{ESI+}) \) 242; UV: \( \lambda_{\text{max}}(\text{pH7}) \) 278nm, \( \lambda_{\text{max}}(\text{pH1}) \) 278nm, \( \lambda_{\text{max}}(\text{pH13}) \) 267nm. As expected these UV profiles were the same as those obtained for the commercial 3-methylcytidine.

**1-[\text{14}C],2-Oxoglutarate assays** - This assay is based on the method used to measure \( ^{14}\text{CO}_2 \) release by \( \alpha \)-ketoisocaproate oxygenase (25). Standard assay conditions comprised of a total volume of 100µL, 50mM TRIS, pH7.5, 4mM ascorbate, 160µM 2OG (2.5% 1-[\text{14}C]), 80µM (NH\(_4\))\(_2\)SO\(_4\),FeSO\(_4\).6H\(_2\)O, 0.48mg mL\(^{-1}\) catalase, 12.5µM AlkB. Generally two stocks were made, one total volume 25µL contained AlkB and Fe\(^{II}\), the second total volume 75µL contained all other reagents. Assays were started by the addition of the Fe\(^{II}\), AlkB stock to the other stock. A tube containing 200µL hyamine hydroxide was added and the vial sealed. The assays were incubated at 37°C for 5 mins and quenched with 20% (v/v) trifluoroacetic acid (300µL). They were then left on ice for 30 mins to collect \( ^{14}\text{CO}_2 \) gas, before the hyamine hydroxide was removed and treated with scintillant for counting (Beckmann, LS6500). Assay points were performed in triplicate unless otherwise stated, values quoted are an average with standard deviation given as the error. DNA oligomers were incubated at a final concentration of 10µM. Nucleoside and base substrates were dissolved at 20mM in DMSO, these were added to a final concentration of 400µM, AlkB activity was not affected by the presence of 2% (v/v) DMSO. For the inhibitor, replacement of ascorbate and variation of 2OG concentration assays, 800µM 1-methyladenosine was present. Inhibitors were assayed at 5 concentrations around the approximate IC\(_{50}\)
value, the maximum concentration of inhibitor tested was 4mM. Initial rate was plotted against inhibitor concentration in an Excel worksheet and a curve was fitted to the data, the equation for the curve was solved for half the rate in the absence of inhibitor to give an approximate IC\textsubscript{50} value. For assays in which Fe\textsuperscript{II} concentration was varied with inhibitor concentration held at IC\textsubscript{50}, Fe\textsuperscript{II} was added to a final concentration of 40, 80, 160, 250 and 500\(\mu\)M. For the assays using alternative reducing agent a 100mM stock of reducing agent was made and added to a final concentration of 4mM. For variation of ascorbate concentration assays, ascorbate was added to a final concentration of 0, 0.25, 0.5, 1 and 4mM. For variation of 2OG concentration assays, 2OG was added to a final concentration 50, 75, 100, 125, 150, 175, 200, 250, 300, 400, 500 and 700\(\mu\)M, these assays were single points and carried out at both 80 and 250\(\mu\)M Fe\textsuperscript{II}. Graphs showing variation of ascorbate and 2OG concentration were plotted in SigmaPlot.

**HPLC/ LCMS assays** - HPLC was carried out using a Synergi C-18 Hydro column, (250\(\times\)4.6mm 4\(\mu\)m) from Phenomenex, the elutant was analysed using a Photodiode Array Detector. All solvents were filtered through 0.2\(\mu\)m filters and sparged with He (g) at 100mL min\(^{-1}\) for 20mins before use. Prior to injection 1% (v/v) acetic acid was added to samples and they were centrifuged at 13000rpm for 10mins. The column was run at 0.8mL min\(^{-1}\) in 50mM ammonium acetate pH6.4, 2% acetonitrile for 25mins, then a gradient was run to 30% acetonitrile over 5mins and held there for 10mins. AlkB assays for HPLC were carried out using the same concentrations of reagents as above, except that no radiolabelled 2OG was present and they were quenched with 1 volume of MeOH after 40 minutes and placed on ice to precipitate the protein. HPLC runs for 1-methyladenosine, 1-methyl-2′-deoxyadenosine, 3-methylcytidine and 3-methyl-2′-deoxycytidine assays were also analysed with some
demethylated nucleoside added to confirm that product would be detected if
demethylation had occurred. The same conditions were used for the LCMS assays
with products being analysed with a Micromass ZMD mass spectrometer ESI(+/−).

**Factor inhibiting HIF assays** - Factor inhibiting HIF (FIH) was purified and assayed
as described previously with the C-terminal fragment of HIF1α(775-826) as a GST
fusion protein used as a substrate (21).

**Synthesis of inhibitors** - N-Oxalylglycine, N-oxalyl-4S-alanine, N-oxalyl-4R-alanine
(26) and 2-hydroxyglutarate (27) were prepared according to literature procedures. N-
Thiono-oxalylglycine was prepared as follows: The dimethyl ester of thiono-
oxalylglycine was prepared as reported (28), and subsequently deprotected to give the
desired product (26) (1.7mmol, 85%) as a yellow solid, mp 105-106 °C; νmax (NaCl, MeOH)/cm⁻¹ 1730, 1697 (C=O); δH(200 MHz; DMSO-d6) 4.27 (2 H, d, 3JHH 6.0, CH2), 10.9 (1 H, br t, NH); δC(50 MHz; DMSO-d6) 163.4, 169.4, 189.5
(C=O, C=S); m/z(AP–) 162 (M – H⁺, 30%), 118 (M – H⁺ – CO₂, 100%); HRMS 161.9861 calculated for M – H⁺ (C4H4NO₄S), 161.9861 found.

(±)-2-Mercaptoglutarate was prepared according to the literature procedure (29). 2-
Acetylsulphanyl-pentanedioic acid diethyl ester was prepared as reported (29,30) then
deprotected by mixing 10mmol (2.62g) of 2-acetylsulphanyl-pentanedioic acid
diethyl ester with 40ml of 2M aqueous sodium hydroxide solution to give 1.37g
(8.3mmol, 83%) of (±)-2-mercaptoglutarate as a colourless solid (spectroscopic data
not previously reported), mp 95-96 °C; νmax (NaCl, MeOH)/cm⁻¹ 1708 (C=O); δH(200
MHz; DMSO-d6) 1.70-2.13 (2 H, m, SCHCH₂CH₂), 2.35 (2 H, t, 3JHH 7.5, SCHCH₂CH₂), 3.37 (1 H, t, 3JHH 7.5, SCH), 12.59 (2 H, br s, COOH), SH signal
probably obscured by DMSO; δC(50 MHz; DMSO-d6) 31.0, 32.0 (CH₂CH₂, SCH
signal obscured by DMSO resonance), 174.5, 174.8 (C=O); m/z (AP−) 163 (M − H+,
100%).
3. Results and discussion

*Expression and purification* - The *alkB* gene was cloned from *E. coli* and inserted into the pET-24a(+) vector. A three column protocol was developed for purification of AlkB based on cation exchange and gel filtration chromatography, giving the desired protein at >95% purity by SDS-PAGE analysis.

*Substrate assays and structural recognition* - AlkB activity was analysed by incubating AlkB with 1-[^14]C]-2OG and measuring the release of[^14]CO₂ upon formation of succinate (25). The results were consistent with the conclusions of Trewick *et al.* and Falnes *et al.* that AlkB is a 2OG dependent Fe^{II} dioxygenase (17,18). Several oligonucleotides including poly(dA) and poly(dC) were reacted with the S_N2 methylating agent MMS. When assayed with AlkB, the unmethylated oligonucleotides did not increase 2OG turnover significantly above the uncoupled rate (Table 1). However, the methylated oligonucleotides gave rise to a significant increase in decarboxylation of 2OG by AlkB. The methylated oligomers used to ascertain the identity of the substrates of AlkB (18) are not precisely defined in a chemical sense as they are made by non-specific methylation of an oligomeric substrate. Consequently, monomeric, potential substrates were assayed to probe the structural features of DNA that are required for AlkB catalysis.

Various methylated bases, nucleotides and nucleosides were assayed as potential substrates for AlkB, including those with the 1-methyladenine and 3-methylcytosine bases that are substrates for AlkB when incorporated into oligomeric DNA (Table 2) (17,18). Incubation with 1-methyladenosine or 1-methyl-2′-deoxyadenosine was found to induce up to a seven-fold increase in 2OG turnover. Whilst 3-methylcytidine and 3-methyl-2′-deoxycytidine only brought about a two and three fold increase
respectively. This suggests that 1-methyladenine rather than 3-methylcytosine lesions may be preferred substrates for AlkB. An HPLC based assay was developed to investigate the production of nucleosides from their methylated counterparts by AlkB. Neither analysis by photodiode array HPLC or by ESI-LCMS led to the detection of any demethylated nucleoside for the incubation of either 1-methyladenosine, 1-methyl-2′-deoxyadenosine, 3-methylcytidine or 3-methyl-2′-deoxycytidine with AlkB. Thus, unlike the situation with oligomeric DNA the turnover of 2OG and hydroxylation of the prime substrate appear to be predominantly uncoupled in the presence of nucleoside substrate analogues. This is of interest with regard to substrate recognition by AlkB. Crystallographic, spectroscopic and solution studies of other 2OG oxygenases have indicated that binding of the prime substrate to the enzyme complex is required to initiate dioxygen binding and subsequent reaction of 2OG (31-33). It appears that 1-methyladenosine and 3-methylcytidine nucleosides are recognised by the AlkB active site and can stimulate reaction of 2OG with dioxygen, but the reactive oxidising species, believed to be [Fe^{IV}=O ↔ Fe^{III}-O'], does not react with the methyl group. It may be that when the requisite connections are not made to the DNA oligomer the reactive oxidised species is not positioned correctly to effect hydroxylation. To effect further 2OG catalysis the original Fe^{II} center must be regenerated by an alternative pathway possibly involving ascorbate (see below). To further investigate the structural motifs recognised by AlkB, assays with 1-methyladenine and various non-methylated bases and nucleosides were carried out (Table 2). The rate of 2OG turnover remained consistent at the level observed in the absence of substrate when AlkB was incubated with adenosine, 2′-deoxyadenosine or adenine, i.e. in the absence of the 1-methyl group. For both 1-methyladenine, 1-methyl-2′-deoxyadenosine and 1-methyladenosine there was an increase in the rate of
2OG turnover, indicating that the 1-methyl group is an absolute requirement for stimulation of the reaction between 2OG and O₂. 2OG conversion was lower for 1-methyladenine than for 1-methyladenosine and 1-methyl-2'·deoxyadenosine, indicating that the presence of a sugar moiety improves substrate recognition by AlkB, possibly by direct contacts between the enzyme and the sugar giving rise to tighter enzyme/substrate analogue binding. Similarly, cytidine, 2'·deoxycytidine and cytosine all failed to bring about an increase in the rate of 2OG conversion, indicating that AlkB activity is stimulated by binding the methyl group of 3-methylcytidine nucleosides.

Stimulation of 2OG oxidation without coupling to hydroxylation of the prime substrate has previously been observed for procollagen prolyl-4-hydroxylase with substrate analogues (34). Further, mutagenesis studies on deacetoxycephalosporin C synthase (DAOCS) have resulted in an uncoupling of 2OG turnover and prime substrate hydroxylation (35). For procollagen prolyl-4-hydroxylase the uncoupled oxidation of 2OG has been linked to the requirement for ascorbate as well as Fe^{II} and 2OG. It has been proposed that an ascorbate molecule regenerates the Fe^{II} center in the event of uncoupled turnover of 2OG to the ferryl intermediate and succinate (36).

Role of ascorbate - Uncoupled 2OG turnover by AlkB was found to be highly dependent on ascorbate both in the presence and absence of 1-methyladenosine, but more so in the former case (Fig. 2). However, the amount of ascorbate required for optimal uncoupled 2OG turnover was far in excess of stoichiometry to 2OG. The role of ascorbate was investigated further by attempting to replace it in the assay with alternative reducing agents (Table 3). Activity was maintained with D-isoascorbate and dithiothreitol, the latter giving ca. 90% of the activity with L-ascorbate. All other agents tested resulted in a significant loss of activity, though, when compared to the
rate of reaction in the absence of reducing agent, \(\beta\)-mercaptoethanol gave a moderate increase in activity, whilst addition of dithionite and 4-nitrocatechol brought about a reduction in rate.

For prolyl-4-hydroxylase, in the presence of poly(L-proline), (a substrate that stimulates uncoupled 2OG turnover but is not hydroxylated) a stoichiometric reaction of 2OG with disappearance of ascorbate has been reported (36). Recent work on anthocyanidin synthase (ANS), a 2OG dependent oxygenase involved in the biosynthesis of flavonoids in plants, has suggested that an ascorbate molecule may bind at the active site in the presence of the prime substrate and be involved in the catalytic cycle (37). Elucidation of the exact role of ascorbate in AlkB catalysis will require further work. However, the observation that it can be replaced by dithiothreitol suggests that its role may not be entirely specific.

Inhibition of AlkB - During the course of this work it was found that 2OG concentrations greater than 200\(\mu\)M caused a reduction in the initial rate of 2OG turnover (Fig. 3). This inhibition was unaffected by an increase in \(\text{Fe}^{II}\) concentration and the same pattern was observed at both 80\(\mu\)M and 250\(\mu\)M \(\text{Fe}^{II}\). Therefore, AlkB inhibition at high 2OG concentrations is unlikely to be a result of \(\text{Fe}^{II}\) chelation by 2OG. The 2OG dependent dioxygenases DAOCS and thymidine-7-hydroxylase have also been observed to be inhibited by high concentrations of 2OG (33,38). Enzyme inhibition by high substrate concentrations is often thought to be a result of two molecules of substrate binding to the enzyme to produce an inactive complex (39). It is possible, though unlikely, that inhibition of AlkB by 2OG has physiological significance. The inhibition of AlkB by high concentrations of 2OG prompted us to investigate the inhibition of AlkB by a variety of structural analogues of 2OG.
The ability to selectively inhibit enzymes that repair alkylation damage to DNA could potentially allow a reduction in the amount of cytotoxic alkylating agents used in cancer chemotherapy. Various compounds were therefore assayed as inhibitors of AlkB (Table 4). N-Oxalylglycine differs from 2OG by the replacement of the 3-CH$_2$ with an NH and has previously been reported to be a competitive inhibitor of both the procollagen (26) and the HIF hydroxylases (20,21). N-Oxalylglycine was found to be a moderate inhibitor of AlkB with an IC$_{50}$ of 0.70mM. The 2-thione derivative of N-oxalylglycine gave a similar IC$_{50}$ of 0.81mM. Both $R$- and $S$- enantiomers of N-oxalylalanine were then tested as inhibitors. Neither stereochemistry was found to be a good inhibitor, with IC$_{50}$ values of 2.4 and 3.3mM for the $S$- and $R$- enantiomers respectively, both significantly higher than that for N-oxalylglycine. This observation implies that space within the 2OG binding pocket of AlkB may be limited compared to some other 2OG oxygenases. N-Oxalyl-4$S$-alanine was also found to be a better inhibitor than the $4R$ of procollagen prolyl-4-hydroxylases (26). Inhibitors in which the 2-keto of 2OG was replaced with a thiol or an alcohol were then tested. As these compounds lack the carbonyl group that reacts with dioxygen it is theoretically impossible for them to undergo nucleophilic attack by an activated dioxygen molecule. The C-2 thiol, (±)-2-mercaptoglutarate had the lowest IC$_{50}$ (0.12mM) of the 2OG analogues tested; in contrast the C-2 alcohol showed no inhibition up to a concentration of 4mM. The significant difference between inhibition by the alcohol and the thiol is interesting and is possibly a result of the high affinity of the thiol for Fe$^{II}$. Changing the concentration of Fe$^{II}$ had no effect on inhibition by (±)-2-mercaptoglutarate, indicating that simple solution based Fe$^{II}$ chelation was not responsible for inhibition. It is also noteworthy that, during catalysis by isopenicillin N synthase, an oxidase with a close structural relationship to the 2OG oxygenases,
ligation of its thiol substrate \((L)\-\delta\-\(\alpha\)-aminoadipyl)-(L)-cysteinyl-(D)-valine to the Fe\(^{II}\) center is a key step in catalysis (40). Thiols are present in a number of pharmaceuticals, therefore it may be of interest to pursue their use in attempts to prepare generic templates for inhibition of 2OG dependent oxygenases.

Recently, it has been proposed that the naturally occurring flavonoid quercetin, (a compound used in dietary supplements) can regulate the hypoxic response by inhibition of the HIF hydroxylases involved in the degradation of the HIF-1\(\alpha\) protein (41). HIF-1\(\alpha\) is hydroxylated by members of the Fe\(^{II}\), 2OG dependent dioxygenase family at an asparagine residue by FIH and two proline residues by the prolyl hydroxylase (PHD) enzymes. Quercetin is a good iron chelator and its regulatory role could in part be due to its effect on iron concentrations and consequently the activity of the Fe\(^{II}\) dioxygenases. Under the standard AlkB assay conditions, quercetin was found to have an IC\(_{50}\) of 0.08mM for inhibition of 2OG turnover by AlkB. However, AlkB activity could be returned to normal levels by addition of excess Fe\(^{II}\) (160\(\mu\)M) (data not shown). This indicates that there is unlikely to be any specific and tight binding interaction between quercetin and AlkB. However it may still be possible to modulate the activity of AlkB using non-specific iron chelators. For comparison the effect of quercetin on the asparagine hydroxylase FIH was also explored. Quercetin was found to inhibit FIH with an IC\(_{50}\) of 0.6mM. Increasing the Fe\(^{II}\) concentration failed to return FIH activity to the normal rate, indicating a specific interaction between FIH and quercetin. The specificity of quercetin for FIH inhibition over that of AlkB could aid the design of inhibitors that specifically inhibit members of the 2OG dependent family of dioxygenases involved in the hypoxic response or in repair of alkylated DNA. The inhibition of FIH by quercetin may also reflect a role for flavonoids in human metabolism, but to conclude such at present is premature.
References


**Foot Notes**

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The abbreviations used are: 2OG, 2-oxoglutarate; MMS, methylmethane sulphonate; FIH, factor inhibiting HIF; HIF, hypoxia-inducible factor; ANS, anthocyanidin synthase; DAOCS, deacetoxycephalosporin C synthase; HPLC, high pressure liquid chromatography; MS, mass spectrometry; ESI, electro-spray ionization; LCMS, liquid chromatography mass spectrometry; poly(dA), polydeoxyadenine; poly(dC), polydeoxycytosine.
<table>
<thead>
<tr>
<th>Oligomeric ssDNA substrate</th>
<th>2OG turnover (pmoles/s)</th>
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<tbody>
<tr>
<td>27mer</td>
<td>1.39±0.4</td>
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<tr>
<td>Me27mer</td>
<td>2.76±0.2</td>
</tr>
<tr>
<td>33mer</td>
<td>0.92±0.1</td>
</tr>
<tr>
<td>Me33mer</td>
<td>2.21±0.3</td>
</tr>
<tr>
<td>poly(dA)</td>
<td>1.55±0.4</td>
</tr>
<tr>
<td>Mepoly(dA)</td>
<td>2.65±0.3</td>
</tr>
<tr>
<td>poly(dC)</td>
<td>0.72±0.3</td>
</tr>
<tr>
<td>Mepoly(dC)</td>
<td>2.00±0.3</td>
</tr>
</tbody>
</table>

Table 1: 2OG turnover by AlkB in the presence of oligomers before and after they had been treated with MMS. The prefix Me indicates the oligomer had been treated with MMS.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>2OG turnover (pmoles/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>1.75±0.1</td>
</tr>
<tr>
<td>1-methyladenosine</td>
<td>12.25±0.4</td>
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<tr>
<td>1-methyl-2’-deoxyadenosine</td>
<td>12.79±0.5</td>
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<tr>
<td>adenosine</td>
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<tr>
<td>1-methyladenine</td>
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<td>adenine</td>
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<td>3-methylcytidine</td>
<td>3.57±0.4</td>
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<td>3-methyl-2’-deoxycytidine</td>
<td>4.98±0.5</td>
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<tr>
<td>cytidine</td>
<td>1.73±0.2</td>
</tr>
<tr>
<td>2’-deoxycytidine</td>
<td>1.68±0.2</td>
</tr>
<tr>
<td>cytosine</td>
<td>1.15±0.6</td>
</tr>
<tr>
<td>6-amino-1-methyluracil</td>
<td>1.01±0.1</td>
</tr>
<tr>
<td>7-methylguanosine</td>
<td>1.67±0.3</td>
</tr>
<tr>
<td>N°-methyladenosine-5’-monophosphate</td>
<td>1.49±0.0</td>
</tr>
<tr>
<td>N²-methylguanosine</td>
<td>1.75±0.2</td>
</tr>
</tbody>
</table>

Table 2: The effect on rate of 2OG turnover by AlkB incubated with various DNA bases, nucleosides and nucleotides.
<table>
<thead>
<tr>
<th>Reducing Agent</th>
<th>2OG turnover (pmoles/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1.32±0.0</td>
</tr>
<tr>
<td>L-ascorbate</td>
<td>11.75±0.1</td>
</tr>
<tr>
<td>D-isoascorbate</td>
<td>12.68±0.4</td>
</tr>
<tr>
<td>dithiothreitol</td>
<td>10.70±1.2</td>
</tr>
<tr>
<td>tris(2-carboxyethyl)phosphine</td>
<td>1.51±0.1</td>
</tr>
<tr>
<td>dithionite</td>
<td>0.56±0.0</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>1.98±0.3</td>
</tr>
<tr>
<td>4-nitrocatechol</td>
<td>0.18±0.1</td>
</tr>
</tbody>
</table>

Table 3: The effect on rate of 2OG turnover by AlkB brought about by replacing ascorbate in the assay with alternative reducing agents
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Structure</th>
<th>IC$_{50}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-oxalylglycine</td>
<td><img src="image1" alt="Structure" /></td>
<td>0.70</td>
</tr>
<tr>
<td>N-oxalyl-4S-alanine</td>
<td><img src="image2" alt="Structure" /></td>
<td>2.39</td>
</tr>
<tr>
<td>N-oxalyl-4R-alanine</td>
<td><img src="image3" alt="Structure" /></td>
<td>3.33</td>
</tr>
<tr>
<td>N-(thioneoxalyl)glycine</td>
<td><img src="image4" alt="Structure" /></td>
<td>0.81</td>
</tr>
<tr>
<td>(±)-2-hydroxylglutarate</td>
<td><img src="image5" alt="Structure" /></td>
<td>-</td>
</tr>
<tr>
<td>(±)-2-mercaptoglutamate</td>
<td><img src="image6" alt="Structure" /></td>
<td>0.12</td>
</tr>
</tbody>
</table>

*Table 4: IC$_{50}$ values and structures of the 2OG analogues tested as inhibitors of AlkB.*
Fig. 1: Oxidative demethylation of DNA by AlkB.

Fig. 2: The effect of ascorbate concentration on uncoupled turnover of 2OG by AlkB.
Filled circles; no substrate: open circles; with 1-methyladenosine.

Fig. 3: The effect of 2OG concentration on the rate of reaction of 2OG with AlkB.
The selectivity and inhibition of AlkB

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