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DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by *O*⁶-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy

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Abstract

*O*⁶-alkylguanine-DNA alkyltransferase (AGT) is a crucial target both for the prevention of cancer and for chemotherapy, since it repairs mutagenic lesions in DNA, and it limits the effectiveness of alkylating chemotherapies. AGT catalyzes the unique, single-step, direct damage reversal repair of *O*⁶-alkylguanines by selectively transferring the *O*⁶-alkyl adduct to an internal cysteine residue. Recent crystal structures of human AGT alone and in complex with substrate DNA reveal a two-domain α/β fold and a bound zinc ion. AGT uses its helix-turn-helix motif to bind substrate DNA via the minor groove. The alkylated guanine is then flipped out from the base stack into the AGT active site for repair by covalent transfer of the alkyl adduct to Cys145. An asparagine hinge (Asn137) couples the helix-turn-helix DNA binding and active site motifs. An arginine finger (Arg128) stabilizes the extrahelical DNA conformation. With this newly improved structural understanding of AGT and its interactions with biologically relevant substrates, we can now begin to unravel the role it plays in preserving genetic integrity and discover how it promotes resistance to anticancer therapies.

Keywords

*O*⁶-alkylguanine-DNA alkyltransferase; AGT; *O*⁶-methylguanine-DNA methyltransferase; MGMT; DNA repair; protein crystal structure

1. Introduction

The genome is under constant attack from both exogenous and endogenous factors. The resulting DNA lesions must be continuously repaired to preserve genomic integrity. Many types of DNA lesions can result, requiring a variety of DNA repair systems, including nucleotide excision repair (NER), base excision repair (BER), and mismatch repair. DNA alkyl

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lesions arise from endogenous sources (such as S-adenosylmethionine) [1], environmental toxins [2], and anticancer chemotherapies [3]. Alkylation at the O^6 -position of guanine can result in G:C to A:T transition mutations [4], which are both mutagenic and cytotoxic. This is because guanine, which normally base pairs with cytosine, will often mispair during replication with thymine when alkylated at the O^6 -position (Fig. 1A). The DNA repair protein O^6 -alkylguanine-DNA alkyltransferase (AGT; E.C.2.1.1.63), also known as O^6 -methylguanine-DNA methyltransferase (MGMT), catalyzes the repair of alkylated guanines by stoichiometrically and irreversibly transferring O^6 -alkyl adducts to an active site cysteine (Cys145 in human AGT) in a suicide reaction, without inducing DNA strand breaks (Fig. 2). Aside from AGT, this type of DNA alkylation removal, sometimes referred to as direct damage reversal, is known in humans for only the ABH2 and ABH3 proteins, both of which are Fe(II)/2-oxoglutarate-dependent dioxygenases that repair the cytotoxic 1-methyladenine and 3-methylcytosine alkylation lesions by coupling oxidative demethylation of these substrates to conversion of 2-oxoglutarate into succinate and CO₂ [5]. AGT-mediated DNA repair differs from other DNA repair pathways, in the following ways: AGT 1) can act alone 2) is both a transferase and an acceptor of the alkyl lesion, 3) is suicidal (inactivated after accepting the alkyl lesion), and 4) mediates stoichiometric repair [6]. These properties make AGT an important target in the design of anticancer strategies.

AGT is widely distributed amongst more than 100 different species, with known examples from Eubacteria, Archaea and Eukarya (Fig. 3) [7]. However, there is no AGT gene in plants, *Schizosaccharomyces pombe*, or *Deinococcus radiodurans* [7]. There are about 25 very highly conserved residues that provide a focus for structural and biochemical studies. Most of these residues have been implicated, through mutagenesis, in DNA binding, facilitation of alkyl transfer, and maintenance of AGT structure [8–18]. Despite significant variance of substrate selectivity across species, AGT is almost exclusively specific for repair of guanines with O^6 -alkyl adducts (Fig. 1B). The only other type of DNA lesion known to be repaired by AGT is O^4 -methylthymine (Fig. 1B). The repair of O^4 -methylthymine by human AGT is very slow and it actually impedes repair by NER, resulting in increased mutation [19,20]. Other AGTs, such as the *E. coli* Ogt, repair O^4 -methylthymine much more rapidly [21]. Human AGT is not limited to O^6 -methylguanine, but can also repair many other adducts at the O^6 -position of guanine, including ethyl-, 2-chloroethyl- and other aliphatic groups [22], and also benzyl- [23] and pyridyloxobutyl- [24] adducts. In fact, perhaps hundreds of AGT pseudosubstrates have been synthesized by various groups (Fig. 4) [25–35].

2. AGT structure

Several X-ray crystallographic and solution structures of unreacted AGT from humans [18, 36], *E. coli* (Ada-C) [37], the archaeobacterium *Pyrococcus kodakaraensis* (Pk) [38], and the thermophile *Methanococcus jannaschii* (MJ1529) [39] have revealed a two-domain α/β fold, with overall dimensions of approximately 20 × 35 × 40 Å for the human protein (Fig. 5A). Comparative analyses of these structures have allowed the identification of conserved structural motifs that are important for DNA recognition, substrate binding and selectivity, the alkyl-transfer mechanism, and the post-alkylation fate of AGT [40]. Although the human, *E. coli*, Pk, and MJ1529 AGTs display only 14% identity and 26% similarity [40], the overall domain structure and fold are almost identical among these four homologs [18,36–39].

The N-terminal domains (approximately residues 1–85) for human, *E. coli*, Pk, and MJ1529 AGTs show no significant primary structural homology, yet they all exhibit a conserved α/β roll structure [39,40]. For all four structures, there is a three-stranded anti-parallel β -sheet followed by either two (human, *E. coli*, MJ1529) or three (Pk) helices. Remarkably, these secondary structures have relatively small variations in their lengths and orientations.

Human AGT contains a Zn(II) ion that is not present in any of the bacterial structures [18]. This Zn(II) ion binds the ligands Cys5, Cys24, His29, and His85 with tetrahedral coordination, and bridges the three strands of the N-terminal β -sheet with the coil immediately preceding the domain-spanning helix. The zinc ligands are completely conserved in the known mammalian sequences, suggesting that this zinc site may be general to higher eukaryotes. The zinc ion likely serves a structural role, and may stabilize the interface between the N- and C-terminal domains by securing the domain-spanning helix to the three strands of the N-terminal β -sheet. Zinc binding causes increased stability of AGT and a small conformational change [41]. This is consistent with small distortions and slightly increased disorder of N-terminal residues in the apo-metal structures [36,42], as compared to the zinc-bound structures [18, 43]. The removal of zinc from AGT does not inactivate this protein, but reduces the rate constant for repair by 60-fold; however, DNA binding is not affected [41].

The topology of the C-terminal domain is absolutely conserved in all known AGT structures. This domain (approximately residues 86–207) is composed of a short two-stranded, parallel β -sheet, four α -helices, and a 3_{10} helix. Contained within this domain are the conserved active-site cysteine motif (PCHR), the O^6 -alkylguanine binding channel, and a helix-turn-helix (HTH) DNA-binding motif. Structures of methylated and benzylated human AGT have established that the active site is located near the recognition helix of the HTH motif [18]. The nucleophilic cysteine, part of the invariant active site PCHR motif of the 3_{10} helix, is located near the bottom of a solvent accessible groove. This groove is approximately 8 Å wide, 9 Å deep and 14 Å long, and defines the O^6 -alkylguanine binding channel. Residues Tyr114-Ala121 and Ala127-Gly136 (human AGT numbering) form the first and second helices, respectively, of the HTH motif. The second HTH helix, called the recognition helix, interacts with DNA. The active site and HTH DNA binding motifs are linked by an “Asn-hinge” (Asn137) (Fig. 3), which stabilizes two overlapping tight turns. This Asn-hinge makes hydrogen bonds with the main chain atoms of Val139 and Ile143 and the thiol of Cys145, and makes contacts through the interdomain cleft with the N-terminal domain, accounting for more than 40% of the buried surface area between the two domains. The geometries of the HTH DNA-binding motif, the Asn-hinge, and the active site helix are essentially identical for all of these structures.

While the C-terminal domain of AGT contains the known necessary residues for DNA binding and alkyl transfer, the function of the N-terminal domain is still not known. In an attempt to better understand the role of the N-terminal domain, the two domains of human AGT have been separately expressed and purified [7]. Interestingly, the C-terminal domain alone retains no activity in the absence of the N-terminal domain and Zn^{2+} . Surprisingly, both in vitro and in vivo experiments suggest that the N-terminal domain has weak AGT activity in the absence of the C-terminal domain. However, neither domain, either separately or together, retained activity in the absence of Zn^{2+} . These data suggest an important structural role for the N-terminal domain in orienting the C-terminal domain for proper catalysis. Notably, two active AGT homologs, cAGT-2 from *Caenorhabditis elegans* [44] and AGTendoV from *Ferroplasma acidarmanus* [45], lack the N-terminal domain. Instead, cAGT-2 and AGTendoV each possess a long C-terminal extension protruding from the AGT domain that is similar to histone 1C and endonuclease V, respectively. These C-terminal extensions may be important for proper catalytic orientation of the active sites in these homologs.

3. AGT substrate binding and nucleotide flipping

Crystal structures of human AGT in the absence of DNA substrates provided a basis for addressing several questions about AGT function, but left some important unanswered questions. For example, by what mode does AGT bind DNA? How does AGT find and recognize an alkylated DNA lesion? Once AGT finds a site of damage, how does it gain access to the alkylated guanine? What is the basis for AGT's substrate specificity? Since the AGT

reaction is stoichiometric, how does it perform its repair in the most efficient manner? Recently, several human AGT-DNA complex crystal structures have become available (Fig. 6). One structure is of a catalytically inactive AGT mutant in a pre-transfer complex with DNA containing the biological substrate *O*⁶-methylguanine (Fig. 6A) [42]. A second structure is of a reaction intermediate consisting of native AGT covalently crosslinked to the N¹,*O*⁶-ethanoxanthosine inhibitor (Fig. 6B) [42]. A third structure is of wild-type AGT in complex with a DNA substrate containing a chemically modified cytosine (N⁴-*p*-xylylenediaminecytosine) (Fig. 6C) [43]. These structures provide a molecular framework that can be used to answer some of the questions about AGT activity and aid in defining a plausible catalytic mechanism.

Mutational data first implicated the HTH motif of AGT as the DNA-binding domain of the enzyme [13,15]. This was later supported by NMR structures of Ada-C, which provided the first direct experimental evidence of HTH-mediated DNA binding for this protein [46], followed by conclusive verification from crystal structures of AGT-DNA complexes [42,43]. HTH motifs are common elements present in roughly one-third of DNA-binding protein families, including DNA repair proteins [42]. Prior to the AGT-DNA complex structures, HTH motifs of DNA repair proteins were thought to interact with the DNA major groove for sequence-specific recognition. Surprisingly, structural data revealed that the HTH motif of human AGT promotes non-classical minor groove DNA binding (Fig. 5B). The well-conserved, small and hydrophobic nature of the recognition helix residues (Ala126, Ala127, Ala129, Gly131, and Gly132) allows this helix to pack closely with the DNA minor groove, minimizes sequence-specific interactions, and may also be advantageous for DNA repair and nucleotide flipping. Thus, AGT-DNA complex structures provided the first examples of HTH-mediated minor groove DNA binding. Other sequence independent DNA-binding proteins with HTH motifs may also exhibit minor groove-mediated DNA binding.

Originally, it was not known how AGT gained access to its alkylated guanine substrate. Initial models of AGT-mediated DNA repair proposed a significant protein conformational change through movement of the C-terminal α -helix to expose the recessed active site cysteine [37]. AGT crystal structures in the absence of DNA substrates, however, indicated that the alkylated guanine may be flipped out of the DNA helix [18,36]. Similarly, NMR structures of Ada-C in complex with DNA provided direct evidence that the conformational change for this protein is minimal upon binding methylated DNA, which suggested that base flipping was required for the repair function [46]. AGT-DNA complex structures confirmed that to gain access to alkylated guanines, AGT rotates the target base out from the DNA base stack and into its active site pocket (Fig. 6) [42,43]. This is not uncommon, since DNA repair proteins often flip target nucleotides to gain access to damaged bases that would otherwise be inaccessible within the DNA duplex structure. DNA binding does not cause much structural change to the human AGT protein itself. However, it does result in significant structural changes to the DNA [42,43]. Specifically, DNA binding causes the minor groove to widen more than 3 Å when compared to B-DNA without distortion, and causes the DNA to bend roughly 15° away from the protein [42]. These changes may help to flip out the damaged base from the DNA helix and to promote AGT cooperative binding to facilitate damage recognition.

Crystal structures of AGT in complex with DNA suggest a mechanism for 3' phosphate rotation-induced nucleotide flipping that involves conserved residues Tyr114 and Arg128 (Fig. 6). The Tyr114 side chain is conformationally restricted, and therefore, by steric clash and charge repulsion, may promote nucleotide flipping via rotation of the 3' phosphate [42]. Nucleotide base flipping with 3' phosphate rotation has been directly observed in the protein-DNA co-crystal structures of other DNA repair proteins, such as the DNA glycosylases AlkA [47], mismatch-specific uracil DNA-glycosylase (MUG) [48], 3-methyladenine-DNA glycosylase [49], uracil-DNA glycosylase (UDG) [50], and human 8-oxoguanine glycosylase

(hOGG1) [51], and the endonucleases apurinic/apyrimidinic endonuclease-1 (APE1) [52] and endonuclease IV [53]. AlkA, UDG, APE1, and endonuclease IV use a single aromatic side chain for phosphate rotation, similar to Tyr114 in human AGT, whereas MUG and hOGG1 use multiple residues for phosphate rotation. This sterically enforced 3' phosphate rotation may be a mechanism general to nucleotide flipping for DNA repair proteins [42].

Arg128, located at the beginning of the recognition helix of the HTH motif, is an “arginine finger” that intercalates via the minor groove between the bases on either side of the substrate guanine (Fig. 6) [42]. This arginine interacts via a charged hydrogen bond with the orphaned cytosine (previously base paired to the flipped-out guanine) to stabilize the extrahelical DNA conformation. It may also be involved in scanning for damaged bases by playing an active role in pushing out bases. Mutations at Arg128 revealed that the length of this side chain is directly proportional to the alkyl transfer rate for methylated duplex DNA, but not for free O^6 -alkylguanine bases [40]. Replacement of arginine with lysine results in a modest loss in activity with methylated duplex DNA, since lysine is long enough to penetrate the base stack but has diminished stacking and hydrogen-bonding capability [40]. The R128L mutant has a five-fold reduction in dealkylation activity [40]. Leucine at this position may pack in the hydrophobic interior of the DNA base stack as seen for UDG [54] and AlkA [47], but it is not able to participate in any hydrogen bonds. Substitutions with even smaller amino acids result in a large loss of activity. The R128G mutation shows less than 0.02% of wild type activity [40]. These mutations have a minimal affect on DNA binding, and therefore do not slow repair through decreased DNA-binding affinity. These observations further support the importance of the role of Arg128 in stabilization of flipped-out bases.

The –PCHR– active site motif (residues 144–147 in human AGT), conserved throughout AGTs, (Fig. 3) [7], contains the active site cysteine (residue 145 for human AGT) (Fig. 2A). This reactive cysteine, located approximately 9 Å from the surface of the protein, [18], has a low pK_a and high reactivity [55]. The cysteine thiolate and methyl group to be transferred are well-positioned for the dealkylation reaction, since they are almost directly opposite one another (Fig. 2) [42]. For the C145S AGT mutant-DNA complex structure, the C145S side chain is approximately 3.4 Å away from the methyl lesion, and rotates more than 90° to accommodate O^6 -methylguanine in the active site (Fig. 2B,6A) [42].

A Glu-His-water-Cys hydrogen bond network (Fig. 2), similar to the Asp-His-Ser catalytic triad of serine proteases, may increase reactivity of Cys145 in AGT [18,40]. This hydrogen bond network is conserved in AGTs, including Ada-C [46]. Like the serine protease catalytic triad, the AGT hydrogen bond network also places its histidine side chain in a hydrophobic environment. It is thought that the low pK_a and high reactivity of Cys145 is due to generation of a thiolate anion via proton transfer through the network. According to this mechanism for the dealkylation reaction, His146 acts as a water-mediated base, while Cys145 acts as the nucleophile. Reactivity may also be promoted by reduction of the negative charge on the repaired guanine through the donation of a hydrogen bond by Tyr114 to N3 of O^6 -methylguanine (Fig. 2) [42].

Structures of AGT in complex with DNA substrates and inhibitors provide a basis with which to better understand recognition and selectivity by AGT. Selectivity for guanine is provided by hydrogen bonds and steric interactions [42]. The extrahelical base fits into a hydrophobic cleft made up of the Met134 side chain and Val155-Gly160 of the active site loop. Cys145 and Val148 carbonyls accept hydrogen bonds from guanine's exocyclic amine. Tyr114 hydroxyl and Ser159 N atoms donate hydrogen bonds to guanine's N3 and O6 atoms, respectively. These interactions provide AGT's selectivity of guanine over adenine. The Gly131 Ca atom is only 3.5 Å away from C2' atom of the ribose, leaving insufficient space for an O2' ribose atom and accounting for the lack of repair of ribonucleotides by AGT. No direct contacts are made with

the extrahelical ribose. Although the basis for recognition of O^4 -methylthymine by AGT has yet to be determined, the structure of AGT in complex with N^4 -*p*-xylylenediaminecytosine suggests that a Tyr114 hydrogen bond to the cytosine O2 atom, may also be present for thymine [43]. However, this structure does not yield insight into protonation of the N3 atom of the damaged base, as for guanine.

Selectivity of O^6 -methylguanine over guanine seems to be based upon hydrophobic interactions [42]. Tyr158 packs against small O^6 -alkyl adducts, and Pro140 can interact with larger O^6 -alkyl adducts [18,42]. There is only about a three-fold increase in affinity for O^6 -methylguanine over guanine [56], which may be the reason that O^6 -alkylguanines are repaired by a unique direct pathway, rather than a more typical base excision pathway. This direct damage reversal may act as a safe-guarding mechanism against the cytotoxic and mutagenic excision of undamaged guanines [42].

While AGT can react with a variety of O^6 -benzylguanine analogs, it prefers para-substituted derivatives to meta-, and it displays poor recognition for ortho-substituted derivatives [27, 57]. The poor recognition for ortho-substituted O^6 -benzylguanine analogs can be explained by the relatively close packing (3.5 Å) of Tyr158 and the recognition helix along the edges of the benzyl group. The Gly160 Ca atom packs against one meta-position at a distance of 4.2 Å. This explains why AGT tolerates a single meta-substituent, but has a decreased rate for two meta-substituents. The para-carbon position is exposed near the inter-domain cleft, and this allows for a variety of bulky substituents at this position. The methyl adduct in the methylated AGT product complex overlays with the corresponding benzylic carbon of the benzyl adduct, but since it is smaller, does not exhibit the extensive hydrophobic packing interactions of the benzyl group [18]. This is consistent with the approximately 2000-fold lower activity of AGT toward O^6 -methylguanine [30].

It is known that for AGT to repair DNA, it must first bind DNA and detect the alkyl lesion. It is not known, however, how AGT efficiently finds sites of damage. Three mechanisms have been proposed for how AGT locates damaged DNA [58]: (1) AGT migrates along DNA and detects lesions by actively flipping out every base into its active site; (2) AGT selectively detects intrahelical lesions resulting from unstable or non-Watson-Crick base pairs; (3) AGT captures a hypothetical transient extrahelical base lesion that would result from the inability of the damaged base to form a stable Watson-Crick base pair with the opposing base of the complementary strand. Unfortunately, there are no direct methods available to distinguish between these scenarios. To better understand base flipping by AGT, Duguid and coworkers used a disulfide cross-linking strategy with a chemically modified cytosine to trap bases in the Ada-C and AGT active sites [58]. Their results suggest that Ada-C only detects and flips out unstable base pairs, whereas human AGT can flip out both stable and unstable base pairs. However, fluorescence and kinetic experiments of repair by human AGT indicate that this protein selectively flips out O^6 -alkylguanines from unstable base pairs, but does not affect normal G:C or A:T base pairs [59]. It has been recently proposed that AGT detects DNA lesions by searching for weakened and/or distorted base pairs rather than for the actual adduct [43].

AGT binds to single- and double-stranded DNA in a highly cooperative manner [56]. This could promote directional bias in repair and may aid the search for damaged DNA through localization of multiple AGT molecules. AGT repairs O^6 -methylguanine lesions at the 5' end of DNA roughly 3.3 times faster than at the 3' end in single stranded oligonucleotides with lesions near both ends [42]. This preference for repair at the 5' end can be eliminated by putting a streptavidin block in the center of the oligonucleotide [42], further emphasizing the bias in the search mechanism. The 3'-to-5' kinetic scanning bias, along with the cooperative DNA binding, suggest efficient AGT binding at the 5' side of a DNA-binding nucleating AGT molecule [42]. This may be through some conformational change, such as further widening of

the DNA minor groove observed in AGT-DNA complex structures [42]. Nucleation by AGT of template DNA soon to be transcribed or replicated slightly ahead of the transcription or replication machinery could result in the cooperative recruitment of other AGT molecules 5' of the initial nucleation site [60]. This would allow for the 3'-to-5' bias and for AGT to remain slightly ahead of transcription or replication machinery. This directional scanning and bias would allow for template DNA lesions to be removed before the polymerases reach them, thereby maximizing the efficiency of repair. By repeating this cycle of cooperative binding and repair, DNA could be constantly refreshed to provide nondamaged template DNA for transcription or replication. Also targeting AGT to regions of DNA where it is most needed would maximize efficient use of AGT and may help to compensate for the suicidal mechanism. However, experiments to determine if AGT can keep up with elongation rates by DNA and RNA polymerases have not yet been done. In support of the model proposed by Daniels et al. [42], genetic and biochemical data indicate that AGT selectively targets DNA undergoing transcription [61].

Once alkylated, mammalian AGT is unstable [62] and is rapidly degraded via the ubiquitin/proteasomal system [63,64]. The cause of ubiquitination is unknown, but may be promoted by alkylated AGT's significant conformational change, which results from a steric clash between the S-alkylcysteine and Met134 [18]. The destabilized protein is rapidly degraded by the 26S proteasome [65]. This rapid degradation may be needed to prevent the inactive alkylated form of the protein from interfering with repair of additional *O*⁶-methylguanine lesions [19]. Inactivated AGT can also prevent transcriptional inactivation of genes that promote cell proliferation by binding the estrogen receptor [66].

The AGT gene contains a number of single nucleotide polymorphisms (SNP), several of which are located in the coding region of the gene: W65C, L84F, I143V, G160R and K178R [67, 68]. Two of these SNPs, I143V and G160R, are near the active site Cys 145. The main chain of Ile 143, for example, makes hydrogen bonds with the Asn hinge (Asn 137), which connects the active site and DNA binding motifs. This could affect the substrate specificity but Ile 143 is replaced by Val in multiple known AGTs (Fig. 3). G160R confers AGT resistance to *O*⁶-benzylguanine [69]. Substitution of glycine for the longer arginine side chain at this position likely obstructs bulky substrate binding through steric or charge clashes. The W65C polymorphism leads to instability of the protein. Trp 65 is located at the interface between N- and C-terminal domains, and it is possible that replacement of the bulky tryptophan side chain with that of the smaller cysteine affects stability by disrupting π -interactions with Tyr 69 of the N-terminal domain and interrupting hydrophobic interactions with the Asn-hinge region in the C-terminal domain [67]. The L84F may affect Zn²⁺ binding, since it is exactly adjacent to the zinc binding ligand His 85. Similar to W65C, this residue is located at the interface between the two domains, but in this case, a large phenylalanine residue is replacing a small leucine residue. AGT structures, therefore, help to explain how these SNPs may have subtle effects on AGT-mediated repair of DNA alkyl adducts, influencing susceptibility to cancer and other diseases.

4. Implications for cancer chemotherapy

In addition to the direct repair of *O*⁶-alkylguanine lesions in DNA, AGT promotes tumor resistance to certain alkylating agents, commonly used in cancer treatments. These fall into two classes. The first are methylating agents such as procarbazine, dacarbazine (DTIC), streptozotocin and temozolomide. The second are chloroethylating agents such as 1,3-bis (2-chloroethyl)-1-nitrosurea (BCNU), ACNU, MeCCNU and CCNU. Both classes cause apoptosis in tumor cells [70–73] but via different mechanisms. Chloroethylating agents form lethal DNA crosslinks via a 1-(3-cytosinyl)-2-(1-guanyl)-ethane bridge. This results from chloroethylation at the *O*⁶-position of guanine, followed by spontaneous cyclization to form

*N*¹,*O*⁶-ethylguanine. This intermediate is unstable and readily reacts with N3 of the opposing cytosine to form the toxic crosslink between N1 of guanine and N3 of cytosine [74–78]. These crosslinks inhibit replication and transcription of DNA and lead to double strand breaks of DNA and apoptosis. Although there are other DNA and protein adducts formed by therapeutic chloroethylating agents, the G-C crosslink is a major factor in the killing of tumor cells. The ability of AGT to repair *O*⁶-chloroethylguanine rapidly and thus prevent the formation of this toxic damage renders the AGT content a critical factor in promoting resistance to these agents. Several studies have documented the importance of AGT in the outcome of therapy with BCNU by showing that a favorable response to treatment is inversely correlated with tumor AGT content [79–81]. Chloroethylating agents have been used for CNS tumors, multiple myeloma, melanoma, lymphoma, gastrointestinal tumors and other solid tumors. Although some remarkable responses have been obtained, their efficacy is limited with less than 20% of patients showing a major response. It is likely that the presence of AGT is a major factor in this limitation.

Therapeutic methylating agents form a wide variety of DNA monoadducts all of which may contribute to cell killing but *O*⁶-methylguanine appears to be of particular importance in leading to cell cycle arrest and apoptosis [73]. Its presence in DNA causes apoptotic cell death via a mechanism that depends on the activity of the mismatch repair (MMR) system [82,83]. One plausible explanation for this is that MMR recognizes *O*⁶-methylguanine:thymine as a mispair but removes the thymine. This causes a futile cycle of nucleotide removal and synthesis that generates single- and double-strand breaks in DNA and leads to apoptosis [84,85]. This explanation was supported by studies showing that *O*⁶-methylguanine was not toxic but highly mutagenic in the absence of MMR due to loss of protein components of MutSα or MutLα. However, studies using point mutations of MSH2 and MSH6 that disrupt the ATP processing ability of MutSα but do not prevent its binding to mismatched base pairs, did not block the apoptotic response to methylation damage [86,87]. Also, MutSα tightly binds *O*⁶-methylguanine:thymine mispairs and the resulting MutSαMutLα complex recruits ATR-ATRIP and activates ATR kinase [88]. These studies indicate that MMR proteins can act to cause cell killing by binding at sites of *O*⁶-methylguanine and signalling the presence of DNA damage. The two mechanisms are not mutually exclusive and both may contribute to the overall effect.

Therapeutic methylating agents are used to treat Hodgkin's disease, brain tumors, melanoma and lymphomas. Temozolomide, with better pharmacokinetic properties than procarbazine or DTIC, was found to be effective in treating anaplastic astrocytomas and glioblastoma multiforme [89–91]. The absence or reduction in AGT activity due to the silencing of the *MGMT* gene by promoter methylation correlates with the efficacy of the treatment of glioblastoma by temozolomide [92,93].

In addition to the studies described above, many experiments using methylating and chloroethylating agents have showed greater cell killing of cultured cells lacking AGT and a better response of human tumor xenografts in nude mice occurs with tumors lacking AGT [22,94–96]. Therefore, a good case can be made for the development of AGT inhibitors that can be used to expand the range and efficacy of agents such as temozolomide and BCNU. A serious drawback with this approach is that alkylating agents are powerful mutagens and, in the absence of AGT, this property, which is known to be associated with the development of secondary tumors, will be enhanced. Thus, approaches that provide tumor specificity to AGT inactivation have particular merit and studies with more general AGT reduction are likely to be justifiable only when no alternatives are available.

Strategies to reduce AGT levels by regulatory approaches such as antisense or ribozymes have been suggested [97,98] and there is a great deal of evidence showing that AGT levels can be

reduced by methylation-induced promoter silencing [92,93]. However, no viable clinical approaches have yet resulted from the development of methods to block AGT synthesis. In contrast, the direct inactivation of AGT has been a very active field, which has resulted in a number of clinical trials.

An important factor in developing anticancer therapies that target AGT is that AGT-mediated repair is stoichiometric. Therefore, since one AGT molecule is needed for each offending alkyl molecule, levels of AGT in cells can be completely depleted by an excess of O^6 -alkylated guanines. Additional factors that make AGT ideal for depletion are that covalent reaction with any O^6 -alkylguanine pseudosubstrate can inactivate it, and once removed, AGT is absent from cells until the synthesis of additional protein. Also, AGT reacts with O^6 -alkylguanine adducts in both free nucleobases and oligomeric deoxyribonucleotides, thus facilitating its inhibition. With this knowledge, several strategies have been used for countering the effects of AGT in cancer chemotherapies.

Attempts to deplete AGT by simply providing larger doses of alkylating agents or combining multiple agents so that the number of O^6 -guanine adducts in DNA exceeds the number of AGT molecules have been largely unsuccessful due to the toxicity [99,100]. Therefore, non-toxic AGT pseudosubstrates have been synthesized. The first of these to have adequate potency was O^6 -benzylguanine, which inactivates AGT through covalent transfer of its benzyl group to the active site cysteine [101,102]. O^6 -Benzylguanine inactivates AGT *in vitro* and *in vivo* and sensitizes tumor cells and xenografts to killing by BCNU or temozolomide [22,94–96]. It is rapidly converted to another equally potent AGT inhibitor, 8-oxo- O^6 -benzylguanine in humans due to its metabolism by CYP1A2 and CYP3A4 [103]. This metabolism renders O^6 -benzylguanine unsuitable for combination with DTIC which is activated by the same enzymes [104]. Adequate doses of O^6 -benzylguanine (c.120 mg/m²) for inactivation of tumor human AGT were readily achievable [105,106]. However, data from ongoing Phase II/III trials suggest that the dose of the alkylating agent that can be given without bone marrow damage is limited due to the lack of specificity of the AGT inhibitor towards the tumor. Despite some responses in these trials, particularly in brain tumors, and in multiple myeloma and subcutaneous T cell lymphomas the value of this therapy has been restricted [103,107–113]. This is most likely due to the lowered dose of BCNU that can be given in combination with O^6 -benzylguanine to avoid myelosuppression and success of these combinations will probably require regional administration approaches [114].

Preliminary results of trials of O^6 -benzylguanine and temozolomide seem more promising. Some responses in adult and pediatric patients with refractory CNS tumors including anaplastic astrocytoma and glioblastoma multiforme were observed [100,115,116]. However, it is clear that improved AGT inhibitors are needed. Since the rate of inactivation of AGT by O^6 -benzylguanine is >10,000 slower than the rate of the normal repair reaction, there is great potential to produce more potent inhibitors. Other O^6 -benzylguanine analogs have been synthesized as potential AGT inactivators, but unfortunately, the potencies of most of these inhibitors show only modest increases and none of these inhibitors exhibit greater potential tumor specificity than O^6 -benzylguanine. One such alternative is O^6 -(4-bromophenyl)guanine (PaTrin-2; Lomeguatrib) (See Fig. 4 (3)), which is in clinical trials in combination with temozolomide in England [95,117–121]. This molecule has a higher reactivity toward AGT and an oral formulation has been developed giving it two potential advantages over O^6 -benzylguanine. Numerous other non-selective AGT inhibitors also based on O^6 -benzylguanine have been described (Fig. 4) but have not been tested clinically. It is noteworthy that O^6 -benzyl-2'-deoxyguanosine was highly active in sensitizing tumor cells and xenografts to BCNU and temozolomide [122]. This is likely to be due to its improved pharmacokinetic properties since it is less potent than O^6 -benzylguanine in the inactivation of purified AGT. Oligonucleotides containing O^6 -benzylguanine [123] or other O^6 -alkylguanines [25] are much

more potent than the corresponding free bases. Another advantage is that multiple AGT-inactivating adducts can be contained in a single oligonucleotide [123] but delivery of these inhibitors presents problems which have not yet been addressed.

The major drawback to the use of O^6 -(4-bromophenyl)guanine or O^6 -benzylguanine is their lack of tumor specificity. Another problem is their poor solubility in aqueous solution. Three approaches to deal with these problems are in progress. Several D-glucose-conjugated inhibitors have been synthesized [124,125]. These water soluble derivatives may be tumor targeted due to increased consumption of glucose by these cells [126] and the up-regulation of the glucose transporter [127]. The most efficient glucose-conjugated inhibitor so far tested is O^6 -4-bromophenylguanine-C8- β D-glucoside [128]. A second tactic has been to generate β -glucuronidase cleavable prodrugs of O^6 -benzyl-2'-deoxyguanosine and O^6 -benzylguanine [129]. These water-soluble derivatives are inactive until activated by β -glucuronidase and may be useful for prodrug monotherapy of necrotic tumors that liberate β -glucuronidase or for antibody-directed enzyme prodrug therapy with antibodies that can deliver β -glucuronidase to target tumor cells. Perhaps the most promising current approach is based on the synthesis of 2-amino- O^4 -benzylpteridine derivatives (See Fig. 4 (2)) which are very potent human AGT inactivators [26]. The most active of these was O^4 -benzylfolic acid. Preliminary studies in cultured cells and in xenografts indicate that O^4 -benzylfolic acid is taken up by the tumor-specific folate receptor system [26], which is an established route to deliver anti-tumor agents selectively [130,131].

Another potential problem in the use of AGT inhibitors such as O^6 -benzylguanine is the selection for AGT mutants that are resistant to inactivation. Alterations of the substrate binding pocket that either reduce the size available to exclude O^6 -benzylguanine or disrupt the hydrophobic binding between O^6 -benzylguanine and the protein are quite readily produced and at least 140 known single point mutations imparting resistance were found. Thirty distinct codons provide sites at which point mutations can cause resistance [94,132,133]. Some of these mutants, particularly those at positions Pro140, Gly156, Tyr158 and Lys165, render AGT almost totally refractory to O^6 -benzylguanine and O^6 -(4-bromophenyl)guanine. When expressed in mammalian cells, these mutant AGT proteins are highly effective in providing protection against BCNU or temozolomide that was not abolished by these drugs. Although they were obtained first in bacteria expressing human AGT, such resistant mutants have now been found in tumor cells treated *in vitro* with BCNU and O^6 -benzylguanine [134,135]. The extent to which newer more potent inactivators such as O^4 -benzylfolate can overcome this problem is not yet clear. O^4 -Benzylfolate is able to inactivate the P140K mutant [26]. This mutant is totally resistant to O^6 -benzylguanine due to the combination of the loss of the interaction of the benzyl group with the Pro140 side chain and the presence of a charged group near to the active site. It is probable that O^4 -benzylfolate binds to additional residues in the AGT active site but these have not yet been determined.

Alkylating agents are toxic to both normal and cancer cells. In normal cells, they are especially toxic to highly proliferative tissues, such as hematopoietic cells and myelosuppression is frequently a dose-limiting toxicity of alkylation therapies. Gene therapy approaches to increase AGT levels in the sensitive bone marrow cells have been suggested [reviewed by [96,136–139]]. The O^6 -benzylguanine-resistant AGT mutants are a logical choice for such studies and may have the additional advantage that selection of stem cells expressing exogenous AGT would be favored in patients treated with O^6 -benzylguanine or O^6 -(4-bromophenyl)guanine. These gene transfers therefore have potential applications not only in the protection of patients treated with chemotherapeutic doses of alkylating agents but for selection of genetically altered stem cells for other nonmalignant diseases. Although some earlier laboratory studies were carried out with the G156A mutant and other AGT mutants with multiple alterations have been

suggested [133], the P140K mutant seems to be the most promising and is the current focus of most of these studies [121,137,139–145].

5. Concluding Remarks

The detailed understanding of base repair processes that is being achieved by integration of structural, biochemical, and biological results is central to an informed basis for understanding and control of genome integrity relevant to more effective cancer interventions [146–148]. The search for inhibitors that are more potent, more readily bioavailable, and can be more specifically targeted to cancer cells continues. Structural analyses of protein interactions with inhibitors and DNA is now becoming possible in solution by small angle X-ray scattering combined with crystallography [149], so progress toward more effective inhibitors is likely to accelerate substantially. Currently, structures of AGT alone, in complex with small molecule inhibitors, and in complex with DNA substrates, provide a detailed knowledge of the active site structure and reaction mechanism. Building upon this knowledge will facilitate efforts to synthesize inhibitors with increased potency and tumor-targeting, making knowledge-based improvements to cancer therapies increasingly effective. More broadly, these DNA base repair structures are valuable as master keys to the understanding and control of human pathologies [150].

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Fig. 1. AGT substrates. (A) Normal guanine:cytosine base pair (left), O^6 -methylguanine:cytosine base pair (middle), and O^6 -methylguanine:thymine base pair (right). (B) O^6 -methylguanine, O^6 -benzylguanine, and O^4 -methylthymine.

Fig 2.

The AGT active site and proposed reaction mechanism. (A) Hydrogen bond network and proposed reaction mechanism for AGT. To facilitate attack at the *O*⁶-alkyl carbon, His146 acts as a water-mediated general base to deprotonate Cys145. The resultant imidazolium ion is stabilized by Glu172. Tyr114 donates a proton to N3 of *O*⁶-methylguanine. (B) Stereo view of the X-ray crystallographic structure of the AGT C145S-DNA complex (pdb 1t38) showing the active site, *O*⁶-methylguanine substrate, and hydrogen bond network.

Fig. 3.

Sequence alignment of AGTs from select organisms. The active site motif and the DNA-binding region of the helix-turn-helix motif are outlined in blue. Human AGT numbering is used. The arginine finger (128), “Asn hinge” (137), and active site cysteine (145) are highlighted in yellow.

Fig. 4.
Selected AGT pseudosubstrates.

Fig. 5. Human AGT X-ray crystallographic structure, zinc site, and DNA binding. (A) Unreacted AGT structure (pdb 1eh6). The N-terminal domain is shown in green, the C-terminal domain is shown in yellow, and the HTH motif is shown in blue. The active site cysteine, arginine finger, "Asn hinge", and zinc ligands are shown in ball-and-stick representation. (B) DNA-bound AGT structure (pdb 1t39). AGT uses the recognition helix of the HTH motif to bind the minor groove of DNA.

Fig. 6. Human AGT substrate binding and nucleotide flipping. X-ray crystallographic structures of AGT in complex with DNA substrates containing (A) O^6 -methylguanine (pdb 1t38), (B) N^1,O^6 -ethanoxanthosine (pdb 1t39), and (C) N^4 -*p*-xylylenediaminecytosine (pdb 1yfh). Tyr114 and Arg128 may promote 3' phosphate rotation-induced nucleotide flipping.