International Journal of
Molecular Sciences

MDPI

Review

O^6-alkylguanine-DNA alkyltransferases in microbes living on the edge: from stability to applicability

Rosanna Mattossovich^§, Rosa Merlo^§, Riccardo Miggiano±, Anna Valenti^* and Giuseppe Perugino^*

^Institute of Bioscience and BioResources, National Research Council of Italy, Via Pietro Castellino 111, 80131 Naples, Italy; rosanna.mattossovich@ibbr.cnr.it, rosa.merlo@ibbr.cnr.it
±Department of Pharmaceutical Sciences, University of Piemonte Orientale, Via Bovio 6, 28100 Novara, Italy; riccardo.miggiano@uniupo.it
* Correspondence: anna.valenti@ibbr.cnr.it; Tel.: +39-081-6132-247 (A.V.)
* Correspondence: giuseppe.perugino@ibbr.cnr.it; Tel.: +39-081-6132-496 (G.P.); Fax: +39-081-6132-646

Received: date; Accepted: date; Published: date

Abstract: The genome of living cells is continuously exposed to endogenous and exogenous attacks, and this is particularly amplified at high temperatures. Alkylating agents cause DNA damage leading to mutations and cell death; for this reason, they also play a central role in chemotherapy treatments. A class of enzymes, defined AGTs, are evolutionarily suitable for the DNA protection caused by alkylating agents, in particular in the recognition and repair of alkylated guanines in O^6-position. The peculiar irreversible trans-alkylation reaction of these enzymes has triggered numerous studies, especially on the human homologue, as from the point of view of basic research, in order to identify effective inhibitors in the fight against cancer, as well as in the modern biotechnology, with the preparation of engineered variants to be used as *protein-tags*. In the last decade, efforts have been made in the characterization of AGTs from (hyper)thermophilic sources as useful model systems that have allowed the clarification of numerous phenomena, common also to mesophilic enzymes. This review traces recent progresses on this class of *thermozymes*, emphasizing their usefulness in basic research and the consequent advantages in *in vivo* and *in vitro* biotechnological applications under non-permissive reaction conditions.

Keywords: thermophilic sources; DNA repair; biotechnological tools, alkylation damage; AGT

1. Introduction

Monofunctional alkylating agents, a class of mutagenic and carcinogenic agents present in the environment, induce DNA alkylation in several positions, as guanine at O^6 (O^6-MG; 6 % of adducts formed), 7-methyl-guanine (N^7-MG; 70 %), and 3-methyl-adenine (N^3-MA; 9 %) [1]. Among them, O^6-alkylation of guanines (O^6-AG) is a cytotoxic lesion: although the specific mechanism of this cytotoxicity is not explicated, it was proposed that the toxic effect occurs after DNA replication, because the O^6-AG incorrectly matches with thymine generating a transition from G:C to A:T [2]. The O^6-MG elimination abnormalities that occur at the time of replication are recognized by the post replication mismatch repair system with potential harmful implications for cell viability. Apart from conventional DNA repair multi-step pathways (as MMR, NER, BER, etc.), alkylated-DNA protein alkyl-transferases (called AGT, OGT, or MGMT; EC: 2.1.1.63) perform the direct repair of alkylation damage in DNA [3, 4]. They represent the major factor in counteracting the effects of alkylating agents that form such adducts [4]. These are small enzymes (17-22 kDa) and widely present in organisms of the three living kingdoms (bacteria, archaea, eukaryotes) but apparently absent from plants, *Schizosaccharomyces pombe*, *Thermus thermophilus* and *Deinococcus radiodurans*. The reaction mechanism of AGTs is based on the recognition of the damaged nucleobase on DNA

[5], followed by a one-step SN_2-like mechanism, in which the alkyl group of the damaged guanine is irreversibly transferred to a cysteine residue in its active site [5-8] (Figure 1, *blue way*).

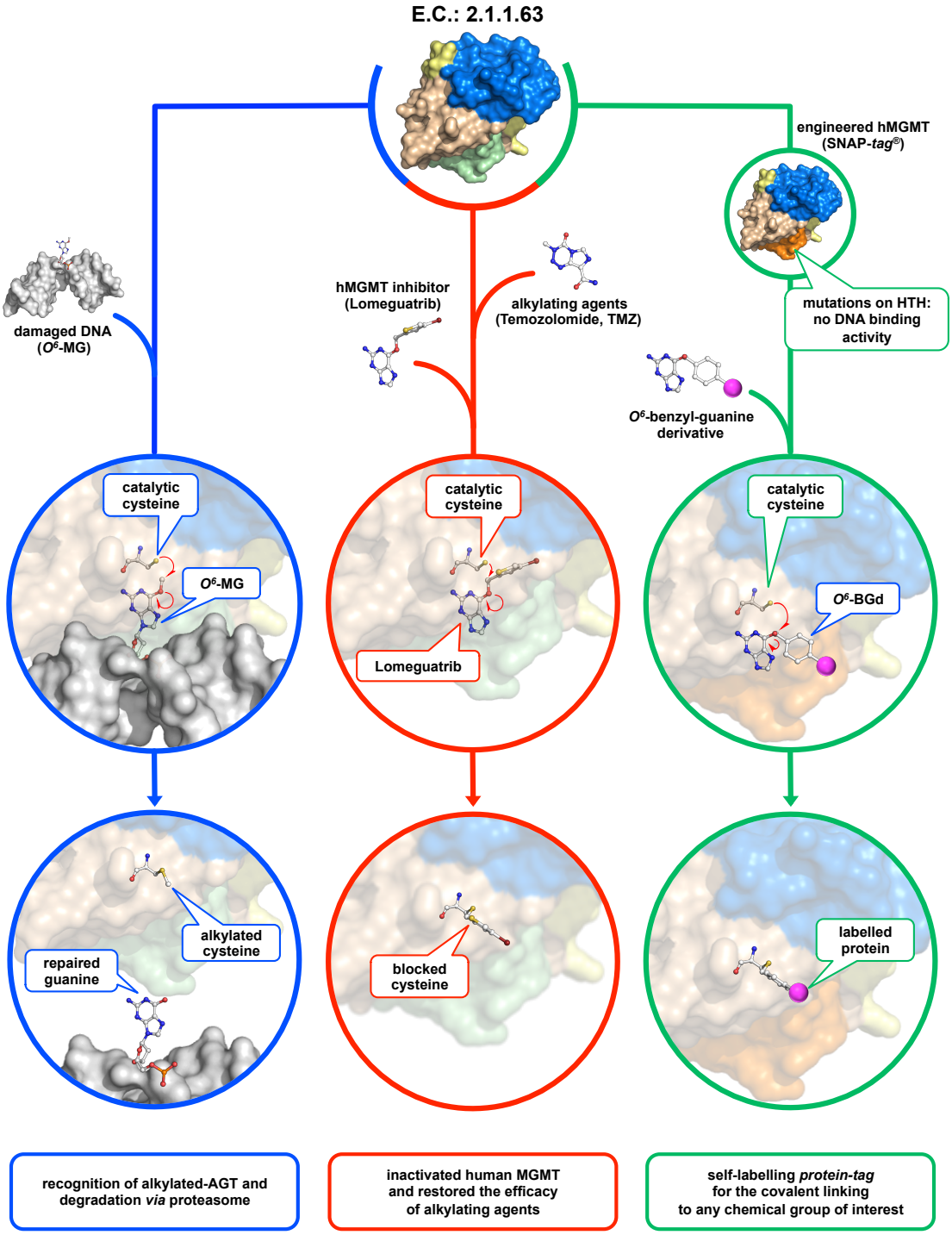

Figure 1. *The AGTs' world*. The peculiar irreversible reaction mechanism of these enzymes plays a pivotal role in the physiological DNA repair (*blue way*), and it has important repercussions in the cancer cells treatment (*red way*) and biotechnological applications (*green way*). Atoms are colored by the CPK convention.

For these reasons, they are also called *suicide* or *kamikaze* proteins, showing a 1:1 stoichiometry of their reaction with the natural substrate. The disadvantage of this elegant catalysis is that, upon alkylation, the protein is self-inactivated and destabilized, triggering its recognition by cellular systems to be addressed to the proteasome [8, 9].

1.1 AGTs as target in cancer therapy

Alkylation damage to DNA occurs in various living conditions, and for this reason the widespread presence of AGT protects cells from killing by means of alkylating agents. However, human AGT (hMGMT) is a *double-edged* sword: on the one hand, it protects healthy cells from these genotoxic and carcinogenic effects; on the other hand, it counteracts alkylating agents-based chemotherapy by also protecting cancer cells from the killing effect of these drugs [10, 11]. Consequently, hMGMT has emerged as a crucial factor in anticancer therapies [12]: in recent years, in fact, an inverse relationship has been discovered between the presence of hMGMT and the sensitivity of cells to the cytotoxic effects of alkylating agents, such as temozolomide (TMZ), in different types of cancer cells, including prostate, breast, colon and lung cancer cells [13].

The resistance to chemotherapy may be reduced by inhibition of these enzymes: as described before, after removing the lesion, the alkylated form of the protein is inactivated and irreversibly addressed towards intracellular degradation pathways. Hence, in order to counteract the action of hMGMT under chemotherapy regimens, a large number of studies have been aimed to the development of hMGMT inactivators to be used in combination with alkylating agents. In view of this therapeutic relevance, much success has been obtained through the design of hMGMT pseudo-substrates, namely the O^6-benzylguanine (O^6-BG) and the strong inactivator O^6-[4-bromothenyl]-guanine (O^6-BTG, Lomeguatrib) [13, 14]. These compounds mimic damaged guanine on DNA and reacts with the protein by the covalent transfer of the alkyl adduct to the active site cysteine residue, causing the irreversible inactivation of the enzyme (Figure 1, *red way*). Therapeutically, O^6-BG is not toxic on its own, but makes cancer cells 2 to 14 times more sensitive to alkylating agents' effects. The oligonucleotides containing more O^6-BG are potent inhibitors and represent a valid alternative to the use of free modified guanines to improve the activity of the alkylating chemotherapy drug in the treatment of some classes of tumors [15, 16, 17].

1.2 AGTs and biotechnology

The specific labelling of proteins with synthetic probes is an important advance for the study of protein function. To achieve this, a way is through the expression of the protein of interest in fusion with additional genetically encoded polypeptides, called *tags*, which mediates the labelling. The first great example of an *autofluorescent tag* was the *Aequorea victoria* green fluorescent protein (GFP) allowing the *in vivo* localization of fusion proteins in cellular and molecular biology fields [18, 19]. Among a*ffinity tags*, of particular importance are the poly(His)-*tag*, the chitin-binding protein, the maltose binding protein [20], the Strep-*tag* [21] and the glutathione-S-transferase (GST-*tag*) [22], which allow fast and specific purification of proteins of interest from their crude biological source using affinity techniques. *Solubilization tags* are especially used to assist the proper folding of recombinant proteins expressed in chaperone-deficient species such as *Escherichia coli*, avoiding protein precipitation: these include the thioredoxin [23] and the poly(NANP).

However, all the *tags* listed above are limited by the fact that each of them can be used for one or some fields of application. The need therefore emerged to somehow use a *universal tag* that could widely cover modern biotechnology.

To overcome these issues, in 2003 the group headed by Kai Johnsson pioneered the use of an engineered hMGMT variant as fusion protein for *in vitro* and *in vivo* biotechnology applications, which led then to its commercialization, namely SNAP-*tag*® (New England Biolabs) [24-27]. They started from the knowledge that hMGMT tolerates very well the presence of groups conjugated to the pseudo-substrate O^6-BG (O^6-BG derivatives): the unusual covalent bond with the benzyl moiety can therefore be exploited for "biotech" purposes (Figure 1, *green way*). Thanks to its small size, SNAP-*tag*® can be fused with other proteins of interest: the expression of the fusion protein inside the cells followed by incubation with opportune fluorescent derivatives leads to *in vivo* labelling and localization of fusion proteins with the probe [24]. The same principle has also been used for the *in vitro* immobilization of tagged fusion proteins: in this case, the O^6-BG is attached to a surface at an adequate distance in order to prevent the enzymatic reaction [28]. This offers a delicate

condition for fixing and disposing in a better orientation a wide range of proteins/enzymes on a surface. The SNAP-*tag*® technology was successfully applied to Surface Plasmon Resonance (SPR) for the covalent immobilization of proteins of interest [29]. Another interesting application of this protein-tag is the possibility to produce new antibody fragments (scFv-SNAP) to be employed in the SPR analysis [30].

Despite the need to use a specific substrate, SNAP-*tag*® offers endless applications: the possibility to covalently link a desired chemical group (conjugated to the O^6-BG) to a protein of interest (genetically fused to it), makes it decidedly advantageous, if compared to traditional *protein-tags* currently in use. Table 1 shows a brief comparison between some examples of *protein-tags* and the SNAP-*tag*® in several application fields.

Table 1. The use of *protein-tags* in some applicative examples.

Applications.	FPs	*affinity tag*	SNAP-*tag*®	notes
in vivo imaging	+[a]	-	+	
substrate utilization	+	-	-	FPs do not need of any substrate for their fluorescence
emission spectra	±	-	+	FPs are in a limited number respect to chemical probes
time-resolved fluorescence	±	-	+	
multi-color fluorescence	±	-	+	for FPs, multi-cloning and expression is necessary
in vitro applications	±	±	+	
variety of chemical group labelling	-	-	+	
pulse-chase analysis	-	-	+	fresh synthetized FPs cannot be efficiently quenched
anaerobic conditions	-	+	+	FPs' fluorophore formation requires oxygen
protein purification	-	+	+	
protein immobilization	±	+	+	immobilized anti-FPs antibody required
pull-down experiments	±	+	+	anti-FPs antibody required

[a] **+**, fully applicable or advantageous; **±**, limited applicability; **-**, not applicable or disadvantageous.

2. Thermophilic and thermostable AGTs

As for organisms living under mesophilic conditions, environmental and endogenous alkylating agents also attack thermophilic and hyper-thermophilic organisms' genomes. Additionally, high temperatures accelerate the process of formation of alkylated bases, leading to DNA breaks [31]. These agents are chemically unstable at the physiological conditions of these organisms, however the collateral decomposition may worse the formation of DNA alkylation products [32]. Thus, the presence of AGTs and methylpurine glycosylases in hyperthermophilic organisms implies they are naturally exposed to endogenous methylating agents [32], supporting the crucial role of AGTs, even if limited information is available on these thermostable proteins [33, 34].

Apart from some studies on Archaea using cell free extracts, few examples of biochemical characterization of AGT from thermophilic sources is that of the enzymes from *Pyrococcus* sp. KOD1 [33] conducted by Imanaka and co-workers, and from *Aquifex aeolicus* and *Archaeoglobus fulgidus* performed by the group of Prof. Pegg in 2003 (Figure 2) [32]. In particular, *A. aeolicus* AGT, whose

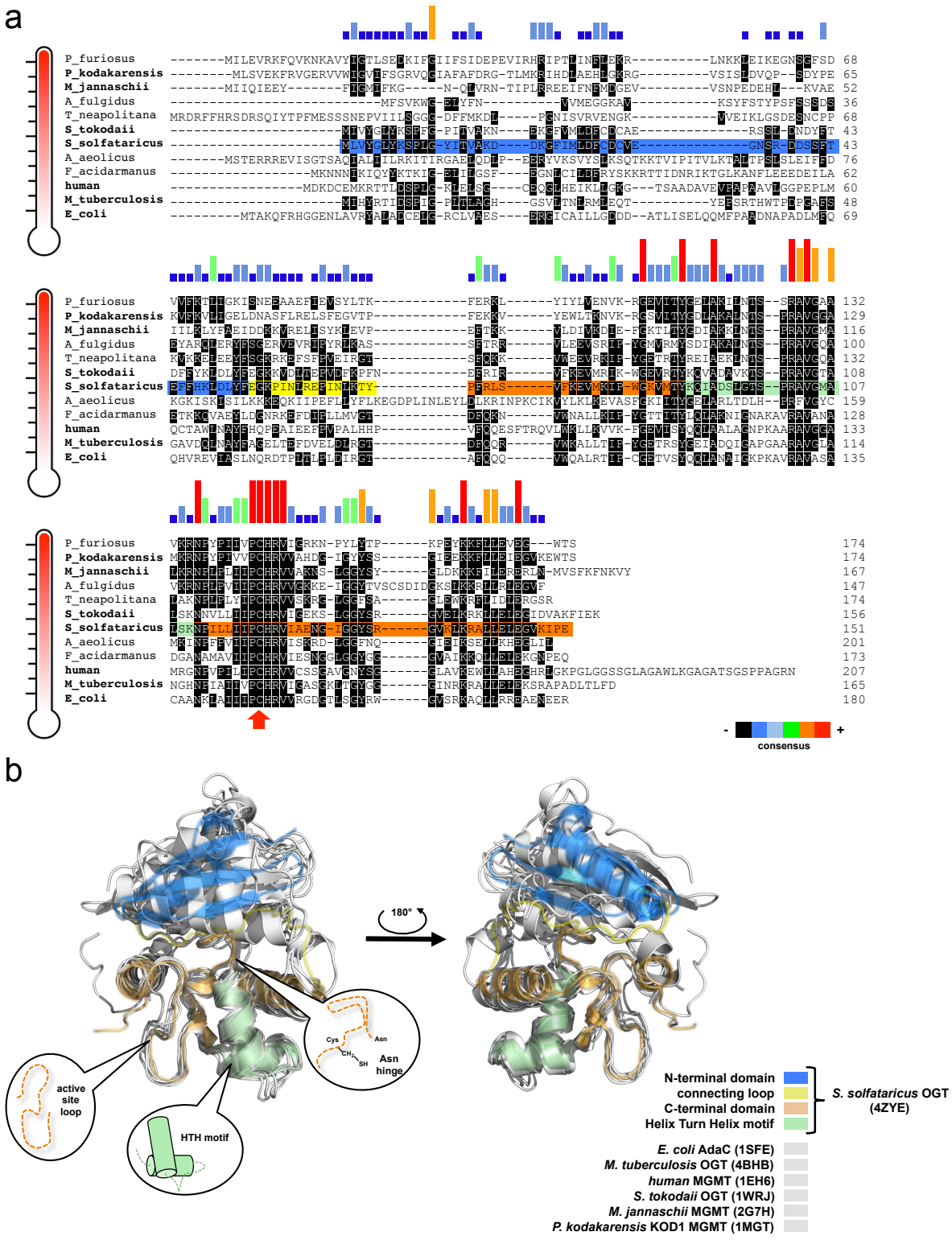

Figure 2. (**a**) Alignment of biochemically and structurally (*in bold*) characterized AGTs. DNA sequences are listed in decreasing order of temperature. The histograms in different colours show the sequence consensus, and the red arrow indicates the highly conserved catalytic cysteine. (**b**) Superimposition of all known AGT structures in their free form (*in grey*). All common domains and elements are coloured only for the *Ss*OGT enzyme. Coloured bars behind the *Ss*OGT sequence in (a) recall the enzyme domains highlighted in the structure and in the legend in (b).

organism was identified as the most primitive bacterium, is closer to the mammalian AGTs than other bacterial homologues in terms of O^6-BG sensitivity [32].

2.1 The common themes in AGTs' tertiary structure and the intrisic factors of stability

Despite the different primary structures (Figure 2a), thermophilic enzymes showed a typical AGT protein architecture, consisting of two domains [35]: a highly conserved C-terminal domain (C_{ter}), surprisingly superimposable for all the AGT structures available on common protein data banks (Figure 2b), and a N-terminal domain (N_{ter}), which is very different among AGTs and whose function is not well fully addressed (likely involved in regulation, cooperative binding and stability [6, 38, 39]). The C_{ter} contains the DNA binding helix-turn-helix motif (HTH), the *Asn hinge*, which precedes the –V/IPCHRVV/I- amino acid sequence of the active site (except the *Caenorhabditis elegans* AGT-2 that has the -PCHP- sequence [36, 37]), and the *active site loop*, responsible for the substrate specificity.

Apart from external factors that could contribute in protein stability, such as the binding to substrate or cofactor [40], to selective ligands [41] and to partner protein [42], the stability and the functional folding of a biological macromolecule is often related to intrinsic factors. A comparative structural analysis performed on AGT proteins whose structures have been deposited in the Protein Data Bank reveals significant differences on the intrinsic structural features that have been considered relevant for thermostability, such as helix capping, intramolecular contacts (hydrogen bonds, ion-pairs), and solvent accessible surface areas. Helix capping plays a central role in the stability of α-helices, due to lack of *intra*-helical hydrogen bonds in the first and last turn [43, 44], and its effect results in an overall structural stabilization of protein folding [45]. By inspecting the crystal structure of the OGT protein from the archaeon *Saccharolobus solfataricus* (hereinafter *Ss*OGT) (PDB ID: 4ZYE), considered here as the thermophilic reference, we verified that the five α-helices composing the protein tertiary structure are characterized by the presence of helix capping, possibly increasing the thermal stability. In particular, the helix *H1* at the N_{ter} is stabilised by a peculiar double serine sequence (S40-S41) and a glutamic acid (E54) at its C_{ter}, the latter is strictly conserved in all AGTs from thermophilic organisms (see Figure 2a). The helix-turn-helix motif (HTH), built on helices *H3* and *H4*, is stabilized at level of *H3* by a highly conserved threonine residue (T89) as N_{ter} capping and a serine (S96), distinctive of *Ss*OGT, as C_{ter} capping. Furthermore, helix *H4* contains two serine-based capping among which the one placed at N_{ter} (S100) is strictly conserved in all thermophilic AGTs and is followed by a proline (P101) that fits well in the first turn of the helix thanks to its own backbone conformation. Finally, the helix *H5* is protected by glutamic acid capping that is present in all the AGTs from different species. Another feature contributing to thermal stability is the solvent-accessible surface area (SASA); indeed, the decrease of SASA and the increase of hydrophobic residues that are buried from the solvent atoms have been considered as stabilized principles for thermostable protein [46]. As described in Table 2, *Ss*OGT shows the smaller total SASA value in line with its exceptional stability, on the contrary OGT from *M. tuberculosis* [38, 47] has the higher value due to the peculiar conformation of both the active site loop and the C-terminal tail that are exposed to the bulk solvent.

Finally, by comparing hyperthermophilic AGTs with the orthologs from mesophilic organisms, in terms of atomic contacts between charged residues as well as intramolecular hydrogen bonds (Table 2), significant differences emerged in the number of charged residues contacts. As expected for thermostable proteins [48], *Ss*OGT, as well as the proteins from *S. tokodaii* and *P. kodakaraensis*, shows a larger number of electrostatic contacts, characterized by higher bond-dissociation energy, with respect to hydrogen bonds for which we did not detect significant differences among the analysed structures, apart from MGMT of *P. kodakaraensis* (*Pk*-MGMT).

Although the number of H-bonds is approximately similar across the AGTs from different organisms, there should be differences in the position-related role of such bonds supporting overall stability of thermophilic variants. With reference to *Pk*-MGMT, Hashimoto and co-workers detected the same number of ion-pairs between the extremophilic protein and *E.coli* AdaC [50]; however, more *intra* and *inter*-helix ion-pairs were found in *Pk*-MGMT. It was suggested an absence of

correlation between ion-pairs' position and stabilization in AdaC, whereas in *Pk*-MGMT the *intra*-helix ion-pairs act on secondary structure elements stabilizing the helices conformations and the *inter*-helix ion-pairs consolidate the inter-domain interactions enhancing the stability of the tertiary structure packing.

Table 2. Comparison of solvent-accessible surface area and intramolecule contacts.

T_{opt}	Organism (PDB ID)	Total SASA (Å)	charged residues contacts	intramolecule H-bonds[a]	Refs
37 °C	*Escherichia coli* (1SFE)	8421.8	74	141	[50]
37 °C	*Mycobacterium tuberculosis* (4BHB)	9535.2	56	143	[38]
37 °C	*Homo sapiens* (1EH6)	8764.3	71	127	[6]
80 °C	*Saccharolobus solfataricus* (4ZYE)	8054.1	94	137	[39]
80 °C	*Sulfurisphaera tokodaii* (1WRJ)	8049.5	124	134	PDB[b]
80 °C	*Methanocaldococcus jannashii* (2G7H)	17770.8[c]	N.D	N.D.	[51]
85 °C	*Pyrococcus kodakaraensis* KOD1 (1MGT)	8302.8	111	157	[49]

[a]Excluding intra-residues H-bonds. [b]https://www.rcsb.org/structure/1wrj. [c]The structure has been solved by means of NMR explaining the high SASA value.

3. The O^6-Alkylguanine-DNA-alkyltransferase from *Saccharolobus solfataricus*

In the last decade *Ss*OGT has been identified and characterized through detailed physiological, biochemical and structural analysis. Due to its intrinsic stability, *Ss*OGT protein has proven to be an outstanding model for clarifying the relationships between function and structural characteristics.

S. solfataricus (previously known as *Sulfolobus solfataricus*) is a microorganism first isolated and discovered in 1980 in the Solfatara volcano (Pisciarelli-Naples, Italy), which thrives in volcanic hot springs at 80 °C and a pH 2.0-4.0 range. In order to protect its genome in these harsh conditions, *S. solfataricus* evolved several efficient protection and repair systems [31, 52]. *S. solfataricus* is highly sensitive to alkylating agent methyl methane sulfonate (MMS), showing a transient growth arrest when treated with MMS concentrations >0.25 mM to 0.7 mM. [31, 52]. Interestingly, while the *ogt* RNA level increased after treatment, the relative enzyme concentration decreases, suggesting its degradation in cells in response to MMS and, in general, to a cellular stresses [52]. Under these treatment conditions, however, the protein level rises after few hours from the treatment, and, in parallel, the growth of *Saccharolobus* starts again [52], indicating a role of ogt in efficient DNA repair by alkylation damage.

3.1 Innovative OGT assays

The attention of researchers, mainly on hMGMT, not only concerns the deepening of critical biological processes, such as DNA repair, but also the development of new and simple intuitive and economic assays, mainly aimed to optimize the inhibitory molecules of therapeutic interest in the cancer treatment. Various assays to measure AGT activity are reported in the literature. The first methods elaborated were based on the use of oligonucleotides carrying radioactive (^{3}H or ^{14}C) O^6-alkylguanine groups. Proteinase K digestion was then carried out and to measure the levels of

marked S-methyl-cysteine in the lysate after an automatic amino acid analyser [53]. A very similar, but simpler and faster radioactive assay has been used: in this procedure, a ^{32}P terminal labelled oligonucleotide contains a modified guanine into a methylation-sensitive restriction enzyme sequence (as *Mbo* I). The AGT DNA repair activity leads to an unmethylated DNA, then target for the restriction enzyme [54]. This procedure was also used by the Ciaramella's group to identify for the first time the activity of *Ss*OGT: this test had the advantage of analysing the fragment digested directly by electrophoresis on polyacrylamide gel [52].

It was therefore improved in terms of precision by the subsequent separation of the oligonucleotides digested by HPLC; however it resulted less simple to perform. The chromatographic separation allowed the calculation of the concentration of active AGT after measuring the radioactivity of the peak corresponding to the digested fragment [55]. Similarly, Luu's group developed the analysis of hMGMT reaction products in 2002, based on HPLC separation. This test investigated the degree of inhibition of oligonucleotides with O^6-MG or O^6-BG in different positions that varied from 3′ to 5′ end and whether they could be used as chemotherapy agents. IC_{50} values were obtained by quantifying the remaining active protein after the radioactive DNA reaction [56].

Although these assays allowed reliable, precise and direct measurements of protein activity, the use of radioactive materials and chromatographic separations make these assays long, tedious and unsafe. An alternative approach was proposed in 2010 by the group of Carme Fàbrega, who set up an assay based on the thrombin DNA aptamer containing a fluorophore and a quencher: the quadruplex structure of this oligonucleotide is compromised if a central O^6-MG is present, hampering the two probes to stay closer. An AGT's repair activity on the oligonucleotide allows the folding of the quadruplex structure and the FRET taking place, resulting in a decrease of the fluorescence intensity [57].

Recently, the introduction of fluorescent derivatives of the O^6-BG (as SNAP Vista Green®, New England Biolabs) makes possible the development of an innovative DNA alkyl-transferase assay. Thus, since AGT covalently bind a benzyl-fluorescein moiety of its substrate after reaction, it is possible to immediately load on a SDS-PAGE: the *gel-imaging* analysis of the fluorescence intensity gives a direct measure of the protein activity, since the 1:1 stoichiometry of protein/substrate (Figure 3). Signals of fluorescent protein (corrected by the amount of loaded protein by coomassie staining analysis) obtained at different times are plotted and a second order reaction rate is determined [38, 39, 47, 52, 58, 59]. This method can be applied to all those AGTs sensitive to O^6-BG, with the exception of the *E. coli* AdaC [60, 61].

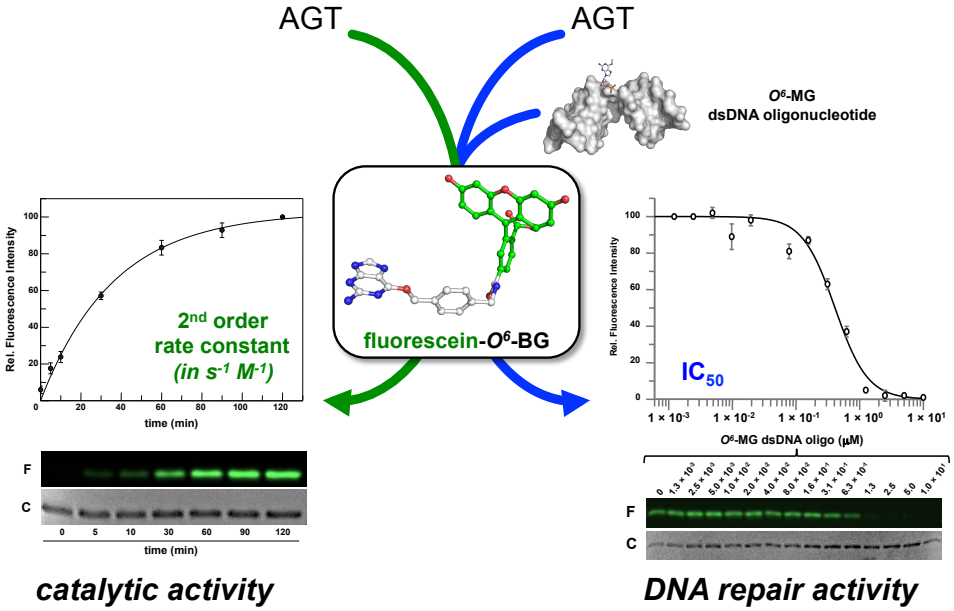

Figure 3. *Innovative fluorescent AGT assay*. The substrate could be used alone for the determination of the AGT catalytic activity, or in combination with a competitive non-fluorescent substrate

(alkylated-DNA). In the latter case an indirect measure of the DNA repair activity on natural substrates is determined (adapted from [62]).

Furthermore, an alkylated dsDNA oligonucleotide can be included in a competition assays with the fluorescein substrate. This non-fluorescent substrate makes lower the final fluorescent signal on *gel-imaging* analysis, depending on its concentration. In this way, it is possible to measure the activity of AGTs for their natural substrate, giving an indirect measure of methylation repair efficiency (Figure 3) [38, 39, 47, 52, 58, 59]. By using this methodology, it was even possible to discriminate the *Ss*OGT activity regarding the position of the O^6-MG on DNA (see below; [39]), in line with previous data on hMGMT [63].

3.2 Biochemical properties of S. solfataricus OGT

The recombinant *Ss*OGT protein, heterologously expressed in *E. coli*, has been fully characterized using the fluorescent assay described in paragraph 3.1 and showed in Table 3. In agreement with its nature, the protein showed optimal catalytic activity at 80 °C, although retaining a residual activity at lower temperatures (Table 2), and in a pH range between 5.0 and 8.0. As for the most part of thermophilic enzymes, *Ss*OGT is resistant over a wide range of reaction conditions, such as ionic strength, organic solvents, common denaturing agents and proteases [52, 58]. Interestingly, chelating agents do not affect the activity of this enzyme: crystallographic data clarified that the archaeal enzyme lacks a zinc ion in the structure [39], whereas this ion is important for the hMGMT correct folding [6].

3.3 Crystal structure of SsOGT

All the steps of the AGTs' activity (alkylated DNA recognition, DNA repair, irreversible trans-alkylation of the catalytic cysteine, recognition and degradation of the alkylated protein) have been structurally characterized. Most knowledge these proteins come from classic studies on hMGMT, as well as the Ada-C and OGT from *Escherichia coli* [5-8, 50], but other AGTs' structures are also available in the Protein Data Bank site (Figure 2a) (http://www.rcsb.org/pdb/results/results.do?tabtoshow=Current&qrid=D3B02F3B).

As described in Figure 1, all AGTs are inactivated after the reaction and degraded *via* proteasome, but in higher organisms, the degradation is preceded by protein ubiquitination [9]. It is common opinion that the recognition of alkylated-AGTs is due by a conformational change: however, data on structure and properties of alkylated AGTs are limited because alkylation greatly destabilizes their folding [39]. The methylated-hMGMT and benzylated-hMGMT 3D structures were only obtained by flash-frozen crystals, showing that alkylation on the catalytic cysteine (C145) induces subtle conformational changes [6, 7, 64]; consequently, these structures might not reflect the

Table 3. Biochemical properties comparison among SNAP-*tag*®, *Ss*OGT wt and the relative H^5 mutant.

		SNAP-*tag*®	***Ss*OGT**	***Ss*OGT-H⁵**
molecular weight (kDa)[a]		23.0	17.0	17.0
T_{opt} (°C)		37.0	80.0	75.0
relative activity	at 25.0 °C	80 %	25 %	**50 %**[b]
	at 37.0 °C	100 %	45 %	**65 %**
	at 80 °C	-	100 %	95 %
catalytic activity at 37 °C		2.8×10^4	2.8×10^3	**1.6×10^4**
pH_{opt}		6.0	7.5	6.0
thermal stability $T_{½}$ (°C)		6 h (37)	3 h (70)	3 h (70)
thermal stability $T_{½}$ at 37 °C		6 h	> 24 h	> 24 h
additives	NaCl	< 0.3 M	> 1.0 M	> 1.0 M
	EDTA	no	yes	yes
	sarcosyl	no	> 0.5 %	> 0.5 %

DDT	yes	no	no

[a]data from [52, 58, 65]. [b]enhancement respect to the *Ss*OGT wt (*in bold*).

physiological conformation of the alkylated hMGMT, since in the crystalline state the protein could not accurately display the conformation adopted in solution [39].

Concerning the interactions with the DNA, *Ss*OGT binds methylated oligonucleotides: the repair activity depends on the position of the alkyl-group [39]: to efficiently repair the alkylated base on dsDNA, the protein requires at least 3 bases from either the 5′ or the 3′ end. This is due to the necessary interactions formed with the double helix. Structural analysis confirmed these data [39].

Considering that AGTs have a highly conserved C_{ter} domain, efforts were put on thermostable homologues of the hMGMT. In contrast to the human counterpart, *Ss*OGT was soluble and relatively stable, thus allowing *in-deep* analysis of the protein in its post-reaction form [39]. Structural and biochemical analysis of the archaeal OGT, as well as after the reaction with a bulkier adduct in the active site (benzyl-fluorescein; [66]), suggested a possible mechanism of alkylation-induced *Ss*OGT unfolding and degradation (Figure 4). Based on their data, Perugino and

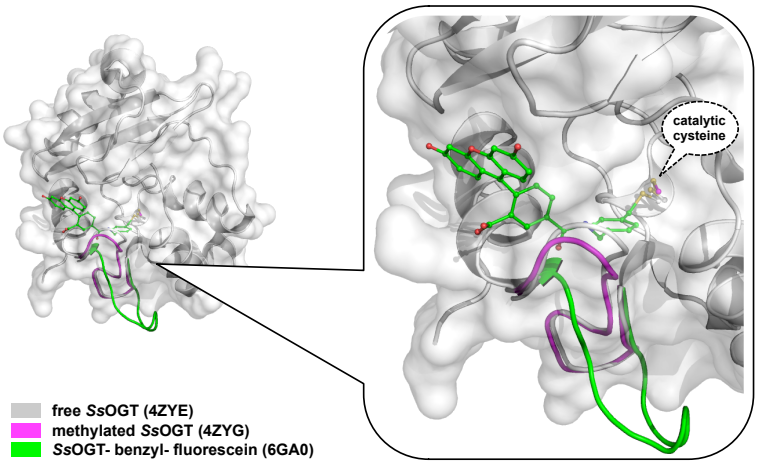

Figure 4. Conformational changes of the *Ss*OGT *active site loop* upon the methyl (*in magenta*) or the benzyl-fluorescein (*in green*) irreversible transfer [39, 66].

co-workers suggested a general model for the mechanism of post-reaction AGTs destabilization: the so called *active-site loop* moves towards the bulk solvent as result of the covalent binding of alkyl adduct on the catalytic cysteine and *the extent of the loop movement and dynamic correlates with the steric hindrance of the adduct* [39, 66] (Figure 4). The destabilization of this protein region triggers the recognition of the alkylated protein to be addressed to degradation pathway.

3.4 Biotechnological applications of an engineered SsOGT, the H⁵ mutant

Apart from new examples of thermostable auto-fluorescent protein (FPs) variants have been exploited in thermophilic microorganisms [67], the general use of most of *protein-tags* is limited only to mesophiles and in mild reaction conditions. As described in paragraph 1.2, the introduction of the SNAP-*tag*® technology enabled a wide *in vivo* and *in vitro* labelling variety for biological studies by fusing any protein of interest (POI) to this *protein-tag* [68]. However, being originated from hMGMT, the application to extremophilic organisms and/or to harsh reaction conditions is seriously limited.

By following the same approach used for the hMGMT by Kai Johnsson, an engineered version of *Ss*OGT has been produced [52, 58]. This protein, called *Ss*OGT-H⁵, presents five mutations in the helix-turn-helix domain, abolishing any DNA binding activity [52]. In addition, a sixth mutation was made: in the *active site loop*, where a residue of serine was replaced by a glutamic acid at position 132 (S132E). This modification increased the catalytic activity of *Ss*OGT [52, 58], as it was

observed in the engineered version of the hMGMT along the SNAP-*tag*® development [24]. H^5 shows slightly lower heat stability in respect to the wild-type protein (Table 3), while the resistance to other denaturing agents is maintained. Moreover, *Ss*OGT-H^5 is characterised by a surprisingly increased catalytic activity at lower temperatures, keeping the rate of reaction to the physiological ones (Table 3) [52, 58]. These behaviours made this mutant a potential alternative to SNAP-*tag*® for *in vivo* and *in vitro* biotechnological applications. The stability against thermal denaturation allowed Miggiano and co-workers to obtain the structure of the protein after the reaction with the fluorescent substrate SNAP-Vista Green®, unrevealing the peculiar destabilization of the *active site loop* upon reaction [66].

3.4.1 *In vitro* thermostable H^5-based chimeras

The *Saccharolobus* OGT mutant has been firstly tested as *protein-tag* fused to two thermostable *S. solfataricus* proteins and in *E. coli* heterologous expressed. The chimeric proteins were correctly folded in *E. coli* cells, and the *tag* did not interfere with the enzymatic activity of the tetrameric β-glycosidase [58], nor with the hyperthermophile-specific DNA topoisomerase reverse gyrase [69-73]. Furthermore, the stability of H^5 made possible a heat treatment of the cell-free extract to remove most of the *E. coli* proteins, as well as the β-glycosidase assay performed at high temperatures, without the need of removing the tag before [58].

3.4.2 Expression in thermophilic organisms models

The applicability of the thermostable *tag* under *in vivo* conditions is fundamental: for this reason H^5 was also expressed in thermophilic organisms, taking advantage of the availability of the fluorescent AGT assay allowed to establish the presence of H^5 both in living cells as well as *in vitro* in cell-free extracts [58, 73]. To address the activity to H^5, it was necessary to choose models in which endogenous AGT activity is suppressed. *Thermus thermophilus* is a *ogt*$^{(-)}$ species, showing only an *agt* homologue (TTHA1564), whose annotation corresponds to an alkyltransferase-like protein (ATL) [74]. ATLs are a class of proteins present in prokaryotes and lower eukaryotes [75], presenting aminoacidic motifs similar to those of AGTs' C_{ter}, in which a tryptophan residue replaces the cysteine in the active site [76]. Like AGTs, ATLs use a helix-turn-helix motif to bind the minor groove of the DNA, but they do not repair it, aim to indeed to recruit and interact with proteins involved in the Nucleotide Excision Repair system [77].

While *T. thermophilus* is a naturally *ogt* knockout organism, *Sulfolobus islandicus* possesses an *ogt* gene very similar to that of *S. solfataricus* and it was silenced by a CRISPR-based technique and was used as a host organism [73].

The fluorescent signal obtained by SDS-PAGE *gel-imaging* analysis revealed that *Ss*OGT-H^5 not only is efficiently expressed in these thermophilic microorganisms, but it also shows that this *tag* was correctly folded and active, demonstrating that H^5 might be used as an *in vivo protein-tag* at high temperatures [58, 73]. Moreover, the use of a fluorescent assay offers another opportunity: as for the human cells, by using different fluorescent substrates, the pathways of POI fused to *Ss*OGT-H^5 could be followed inside living "*thermo* cells".

3.4.3 The ASL^{tag} system

As it is known, most of the biotechnological processes require harsh operational conditions. To overcome the general instability of most of biocatalysts used in the processes, the introduction of immobilised enzymes on solid supports has been helpful for their application [78]. By definition, an immobilized enzyme is a "physically confined biocatalyst, which retains its catalytic activity and can be used repeatedly" [79]. In fact, protein immobilisation offers several advantages, as the catalysts recovery and reuse and the physical separation of the enzymes from the reaction mixture. Currently, different immobilisation strategies are available, from physical adsorption to the covalent coupling [80-83]. However, all these procedures require purified biocatalysts and arduous

techniques, as well as problems related to steric hindrance between the catalyst, the substrate and the solid support, with an increasing of costs and time for the production processes.

The introduction of "cell-based" immobilisation systems resulted in a significant improvement and reduce times and costs of the process. One of the most used display strategies is the simultaneous heterologous expression of enzymes and their *in vivo* immobilisation on the external surface of Gram-negative bacteria cells, by the utilisation of the ice nucleation protein (INP) from *Pseudomonas syringae* [84, 85]. Most recently, the N-terminal domain of INP (INPN) was used to produce a *novel anchoring and self-labelling protein tag* (hereinafter ASL^{tag}). The ASL^{tag} consists of two moieties, the INPN and the engineered and H^5 mutant (Figure 5) [86].

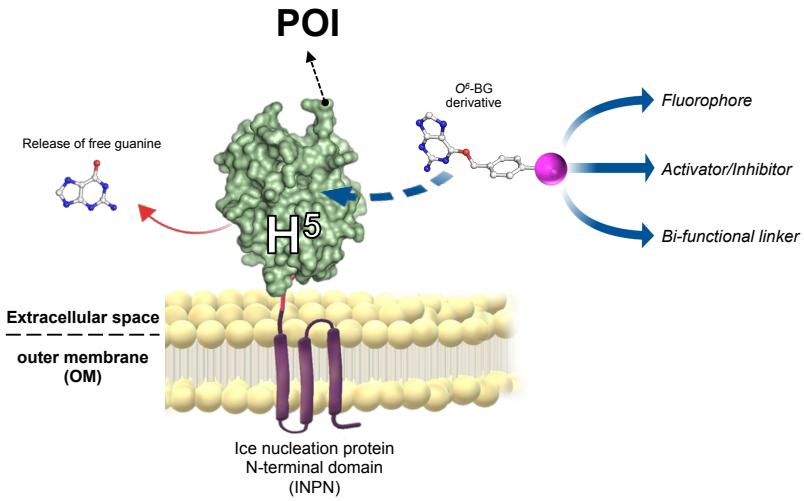

Figure 5. The ASL^{tag} system. A protein of interest (POI) is genetically encoded with the *tag*, which in turn makes it anchored and exposed to the solvent, and contemporary able to covalently link to a desired chemical group (*magenta sphere*) by the activity of *Ss*OGT-H^5 (adapted from [87]).

The former allowed an *in vivo* immobilisation on *E. coli* outer membrane of enzymes of interest, making direct exposition of the enzyme to the solvent possible, leading to a significant reduction of the costs related to purification and immobilization, and to overcoming of problems related to the recovery of enzymes in a process [88]. The latter gives the unique opportunity to label the immobilized enzyme with any desired chemical groups (covalently link to the benzyl-guanine) [25, 58], dramatically expanding biotechnological applications. In particular, it can be possible to modulate the activity of enzymes fused with the ASL^{tag} by introducing activators or inhibitors molecules or connecting them to other biocatalysts by using bi-functional linkers to improve cascade reactions (Figure 5). The ASL^{tag} system allowed the expression and immobilization of enzymes of interest, from monomeric proteins (e.g., the thermostable carbonic anhydrase from *Saccharolobus*, *Ss*pCA) to enzymes having a complex quaternary structure (e.g., the thermophilic *Ss*βGly), without affecting their folding and catalytic activity [86]. Moreover, this system was also useful to stabilise fused proteins, as was the case for *Ss*pCA, which showed an increase in residual activity of up to 30% for a period of 10 days at 70 °C [87], representing a huge advantage in pushing beyond reactions in bioreactors and in the reutilization of biocatalysts.

4. *Pyrococcus furiosus* and *Thermotoga neapolitana* OGT

To date, no *protein-tags* have been applied at extremely high temperatures. Aimed to employ the SNAP-*tag*® technology to hyperthermophilic microorganisms for *in vivo* studies, it has been recently characterized an *O*^{6}-alklylguanine-DNA alkyltransferase from the archaeon *Pyrococcus furiosus* [89]. This extremophilic microorganism was originally isolated from hot marine sediments in Vulcano Island (Italy) [90] with an optimum growth temperature around 100 °C and thriving under extremely harsh conditions. As other thermophilic Archaea, its enzymes are extremely

thermostable and find applicability in various biotechnological applications. Just think to one of the most famous and used enzymes from *P. furiosus*: the DNA polymerase I, also known as *Pfu* DNA polymerase, with a strong 3'-5' *proof-reading* activity [91]. The first demonstration of an OGT activity in *P. furiosus* was in 1998, when Margison and co-workers identified a protein of 22 kDa, whose catalytic activity was abolished by the O^6-BG pseudo-substrate. The PF1878 ORF is relative to a protein of 20.1 kDa: from the primary structure, the relative polypeptide seems to be relatively closed to the MGMT from *Pyrococcus kodakarensis* KOD1 (*Pk*-MGMT) [49, 89, 92]. The extreme thermostability was confirmed by *in vitro* biochemical studies on the heterologous expressed and characterized *Pfu*OGT protein. This enzyme was active on BG-fluorescent substrates, allowing the competitive assay with methylated dsDNA. However, the experiments were performed at 65 °C instead of the standard procedure at 50 °C, as described for *Ss*OGT [39, 58, 59], due to a strong thermophilicity of this enzyme. This behaviour was effectively confirmed by Differential Scan Fluorimetry analysis: the stability of *Pfu*OGT was 80 °C in terms of temperature melting (T_m), if compared with that of *Ss*OGT (68 °C) [89]. It is worth noting that, to obtain the sigmoidal curve for *Pfu*OGT to fit with the Boltzmann equation and calculate the T_m value, a setting of 10 min/°C × cycle have been necessary, whereas the procedure is usually performed at 1 min/°C × cycle [93].

Thermotoga neapolitana is a hyperthermophilic gram-negative bacterium of the order of Thermotogales [94-96], which are excellent models for genetic engineering and biotechnological applications [97-100]. *T. neapolitana* possesses a CTN1690 ORF showing a clear homology of the O^6-alkylguanine-DNA-alkyl-transferase. SDS-PAGE *gel-imaging* analysis on lyophilized *T. neapolitana* cells incubated with the AGT fluorescent substrate showed a strong fluorescent signal with a molecular weight close to that of *Ss*OGT. The observed molecular weight and, above all, the sensitivity to this O^6-BG derivative, led to the cloning and heterologous expression of this protein in *E. coli*, then called *Tn*OGT. [89]. This protein, like most AGTs, has a role in DNA repair and was verified with the competitive fluorescent assay in the presence of methylated dsDNA, as described in Figure 3, leading to a IC_{50} value similar to that obtained with *Ss*OGT. Surprisingly, the enzyme from *T. neapolitana* exhibited a very high activity at low temperatures [89], similar to that shown by the mutant *Ss*OGT-H^5 (Table 3) [52, 58]. This characteristic hindered the determination of the second order constants at temperatures above 50.0 ° C, since its reaction rate went faster than the technical limits of the assay [89]. Superimposition analysis between a *Tn*OGT 3D model and the free form of *Ss*OGT (ID PDB: 4ZYE) revealed in both the presence of a serine residue in the *active site loop* (S132 in *Ss*OGT, see Figure 2a), which was replaced in *Ss*OGT-H^5 by a glutammic acid to improve its activity at lower temperatures. From the superimposition, again, some residues are missing in *Tn*OGT, which play an important role in stabilizing *Ss*OGT. In particular, the ionic interactions that play a crucial role in the stability of the *Saccharolobus* enzyme at high temperatures, such as the pair R133-D27 [39] and the *K-48 network* [59], are mainly replaced by hydrophobic residues in the *Thermotoga* homolog. Evidently, different residues and mechanisms of stabilization contribute to its exceptional catalytic activity at moderate temperatures and the stability at higher ones.

5. Future perspectives

The interest shown for this class of small proteins for decades has led to useful knowledge from basic research to biotechnological applications [101]. Studies on thermophilic AGTs represent a unique opportunity for structural analysis and, in the case of the *S. solfataricus* protein, for the identification of conformational changes after the trans-alkylation reaction, however not possible with mesophilic AGTs, which in the alkylated form are enormously destabilized [6]. These results could have a wide impact especially in medical fields for the design of novel hMGMT inhibitors to be used in cancer therapy [102]. Furthermore, given their small size, thermophilic enzymes are very useful for studying general stabilization mechanisms at high temperatures (as for *Pk*-MGMT and *Ss*OGT), which can then be applied to mesophilic enzymes. Searching for alternative *Ss*OGT homologues was clearly useful, leading to the identification of AGTs more resistant to thermal

denaturation (*Pfu*OGT) or of enzymes with a higher reaction rate at all tested temperatures (*Tn*OGT).

Concerning biotechnology, the modification of AGT in a *protein-tag* is an approach to be considered of general applicability, both following a *rational approach*, by abolishing the DNA binding, as in *Ss*OGT-H[5], and *irrational*, by random mutagenesis and selection of variants for higher catalytic activity, such as SNAP-*tag*® [103], or those modified in substrate specificity, such as CLIP-*tag*®, which is active on benzyl-cytosine (O^2-BC) derivatives [65]. This knowledge could be the starting point to develop new engineered *thermo*-SNAP-*tag*® to be employed in particular biotechnological fields, from *in vivo* studies in (hyper)thermophilic microorganisms (such as the *in vivo* CRISPR-Cas immune system in *P. furiosus* [104, 105]) to industrial processes that require high temperatures or, in general, harsh reaction conditions.

Author Contributions: §R.Ma. and R.Me. equally contributed to the present review article.

Funding: This research was funded by MIUR National Operational Program (PON) Research and Innovation 2014-2020 (CCI 2014IT16M2OP005), European Social Fund, Action I.1 "Innovative Doctorates with Industrial characterization".

Acknowledgments: G.P. would like to thank all the authors, Miss Elena and Elisa Perugino, for their efforts in writing this work, technical assistance, but mainly for human support during the difficult and delicate period of stay-at-home following the COVID-19 outbreak.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AGT	O^6-alkyl-guanine-DNA-alkyl-transferase
CLIP-*tag*®	engineered version of SNAP-*tag*® active on O^2-BC
FP	auto-fluorescent protein
hMGMT	human O^6-methyl-guanine-DNA-alkyl-transferase
MGMT	O^6-methyl-guanine-DNA-alkyl-transferase
O^2-BC	O^2-benzyl-cytosine
O^6-AG	O^6-alkyl-guanine
O^6-BG	O^6-benzyl-guanine
O^6-MG	O^6-methyl-guanine
OGT	O^6-alkyl-guanine-DNA-alkyl-transferase
SNAP-*tag*®	engineered version of hMGMT for biotech purposes

References

1. Liu, L.; Gerson, S.L. Targeted modulation of MGMT: clinical implications. Clin. Cancer Res. **2006**, 12, 328–331.
2. Leonard, G.A.; Thomson, J.; Watson, W.P.; Brown T. High-resolution structure of a mutagenic lesion in DNA. Proc. Natl. Acad. Sci. **1990**, 87, 9573–9576.
3. Drabløs, F.; Feyzi, E.; Aas, P.A.; Vaagbø, C.B.; Kavli, B.; Bratlie, M.S.; Peña-Diaz, J.; Otterlei, M.; Slupphaug, G.; Krokan, H.E. Alkylation damage in DNA and RNA-repair mechanisms and medical significance. DNA Repair **2004**, 11, 1389–1407.
4. Pegg, A.E. Multifaceted roles of alkyltransferase and related proteins in DNA repair, DNA damage, resistance to chemotherapy, and research tools. Chem. Res. Toxicol. **2011**, 24, 618–639.
5. Duguid, E.M.; Rice, P.A.; He, C. The structure of the human AGT protein bound to DNA and its implications for damage detection. J. Mol. Biol. **2005**, 350, 657–666.
6. Daniels, D.S.; Mol, C.D.; Arvai, A.S.; Kanugula, S.; Pegg, A.E.; Tainer, J.A. Active and alkylated human AGT structures: A novel zinc site, inhibitor and extrahelical base binding. EMBO J. **2000**, 19, 1719–1730.
7. Daniels, D.S.; Woo, T.T.; Luu, K.X.; Noll, D.M.; Clarke, N.D.; Pegg, A.E.; Tainer, J.A. DNA binding and nucleotide flipping by the human DNA repair protein AGT. Nat. Struct. Mol. Biol. **2004**, 11, 714–720.

8. Tubbs, J.L.; Pegg, A.E.; Tainer, J.A. 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. DNA Repair **2007**, 6, 1100–1115.
9. Xu-Welliver, M.; Pegg, A.E. Degradation of the alkylated form of the DNA repair protein, O^6-alkylguanine-DNA alkyltransferase. Carcinogenesis **2002**, 23, 823–830.
10. Gerson, S.L. MGMT: its role in cancer aetiology and cancer therapeutics. Nat. Rev. Cancer. **2004**, 4, 296–307.
11. Sabharwal, A.; Middleton, M.R. Exploiting the role of O^6-methylguanine-DNA-methyltransferase (MGMT) in cancer therapy. Curr. Opin. Pharmacol. **2006**, 6, 355–363.
12. Zhong, Y.; Huang, Y.; Huang, Y.; Zhang, T.; Ma, C.; Zhang, S.; Fan, W.; Chen, H.; Qian, J.; Lu, D. Effects of O^6-methylguanine-DNA methyltransferase (MGMT) polymorphisms on cancer: a meta-analysis. Mutagenesis **2010**, 25, 83–95.
13. Kaina, B.; Christmann, M. DNA repair in personalized brain cancer therapy with temozolomide and nitrosoureas. DNA Repair **2019**, 78, 128–141.
14. Khan, O.; Middleton, M.R. The therapeutic potential of O^6-alkylguanine DNA alkyltransferase inhibitors. Expert Opin. Investig. Drugs **2007**, 10, 1573–1584.
15. Paranjpe, A.; Zhang, R.; Ali-Osman, F.; Bobustuc, G.C.; Srivenugopal, K.S. Disulfiram is a direct and potent inhibitor of human O6-methylguanine-DNA methyltransferase (MGMT) in brain tumor cells and mouse brain and markedly increases the alkylating DNA damage. Carcinogenesis **2014**, 35, 692–702.
16. Rabik, C.A.; Dolan, M.E. Molecular mechanisms of resistance and toxicity associated with platinating agents. Cancer Treat. Rev. **2007**, 33, 9–23.
17. Kaina, B.; Margison, G.P.; Christmann, M. Targeting O^6-methylguanine-DNA methyltransferase with specific inhibitors as a strategy in cancer therapy. Cell Mol. Life Sci. **2010**, 67, 3663–3681.
18. Chalfie, M.; Tu, Y.; Euskirchen, G.; Ward, W.W.; Prasher, D.C. Green fluorescent protein as a marker for gene expression. Science **1994**, 263, 802–805.
19. Tsien, R.Y. The green fluorescent protein. Annu. Rev. Biochem. **1998**, 67, 509–554.
20. di Guan, C.; Li, P.; Riggs, P.D.; Inouye, H. Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. Gene **1988**, 67, 1, 21–30.
21. Schmidt, T.G.M.; Koepke, J.; Frank, R.; Skerra, A. Molecular Interaction Between the Strep-tag Affinity Peptide and its Cognate Target, Streptavidin. J. Mol. Biol. **1996**, 255, 753–66.
22. Ren, L.; Chang, E.; Makky, K.; Haas, A.L.; Kaboord, B.; Walid Qoronfleh, M. Glutathione *S*-transferase pull-down assays using dehydrated immobilized glutathione resin. Analytical Biochemistry **2003**, 322, 164–169.
23. LaVallie, E.R.; DiBlasio, E.A.; Kovacic, S.; Grant, K.L.; Schendel, P.F.; McCoy, J.M. (1993) A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. Bio/Technology **1993**, 11, 187–193.
24. Juillerat, A.; Gronemeyer, T.; Keppler, A.; Gendreizig, S.; Pick, H.; Vogel, H.; Johnsson, K. Directed evolution of O^6-alkylguanine-DNA alkyltransferase for efficient labeling of fusion proteins with small molecules *in vivo*. Chem. Biol. **2003**, 10, 313–317.
25. Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. A general method for the covalent labeling of fusion proteins with small molecules *in vivo*. Nat. Biotechnol. **2003**, 21, 86–89.
26. Kindermann, M.; George, N.; Johnsson, N.; Johnsson, K. Covalent and selective immobilization of fusion proteins. J. Am. Chem. Soc. **2003**, 125, 7810–7811.
27. Gronemeyer, T.; Chidley, C.; Juillerat, A.; Heinis, C.; Johnsson, K. Directed evolution of O^6-alkylguanine-DNA alkyltransferase for applications in protein labeling. Protein Eng. Des. Sel. **2006**, 19, 309–316.
28. Hinner, M.J.; Johnsson, K. How to obtain labeled proteins and what to do with them. Curr. Opin. Biotechnol. **2010**, 21, 766–776.
29. Huber, W.; Perspicace, S.; Kohler, J.; Müller, F.; Schlatter, D. SPR-based interaction studies with small molecular weight ligands using hAGT fusion proteins. Anal. Biochem. **2004**, 333, 280–288.
30. Niesen, J.; Sack, M.; Seidel, M.; Fendel, R.; Barth, S.; Fischer, R.; Stein, C. SNAP-tag technology: a useful tool to determine affinity constants and other functional parameters of novel anti-body fragments. Bioconjug. Chem. **2016**, 27, 1931–1941.

31. Valenti, A.; Napoli, A.; Ferrara, M.C.; Nadal, M.; Rossi, M.; Ciaramella, M. Selective degradation of reverse gyrase and DNA fragmentation induced by alkylating agent in the archaeon *Sulfolobus solfataricus*. Nucleic Acids Res. **2006**, 34, 2098–2108.
32. Kanugula, S.; Pegg, A.E. Alkylation damage repair protein O^6-alkyl-guanine-DNA alkyltransferase from the hyperthermophiles *Aquifex aeolicus* and *Archaeoglobus fulgidus*. Biochem. J. **2003**, 375, 449–455.
33. Leclere, M.M.; Nishioka, M.; Yuasa, T.; Fujiwara, S.; Takagi, M.; Imanaka, T. The O^6-methylguanine-DNA methyltransferase from the hyperthermophilic archaeon Pyrococcus sp. KOD1: a thermostable repair enzyme. *Mol Gen Genet*, **1998,** *258*, 69–77.
34. Skorvaga, M.; Raven, N.D.; Margison, G.P. Thermostable archaeal O^6-alkylguanine-DNA alkyltransferases. Proc. Natl. Acad. Sci. **1998**, 95, 6711–6715.
35. Fang, Q.; Kanugula, S.; Pegg, A.E. Function of domains of human O^6-alkyl-guanine-DNA alkyltransferase. Biochemistry **2005**, 44, 15396–15405.
36. Kanugula, S.; Pegg, A.E. Novel DNA Repair Alkyltransferase from *Caenorhabditis elegans*. Env. Mol. Mut. **2001**, 38, 235–243.
37. Serpe, M.; Forenza, C.; Adamo, A.; Russo, N.; Perugino, G.; Ciaramella, M.; Valenti, A. The DNA alkylguanine DNA alkyltransferase-2 (AGT-2) of *Caenorhabditis elegans* is involved in meiosis and early development under physiological conditions. Sci. Rep. **2019**, 9, 6889.
38. Miggiano, R.; Casazza, V.; Garavaglia, S.; Ciaramella, M.; Perugino, G.; Rizzi, M.; Rossi, F. Biochemical and structural studies of the *Mycobacterium tuberculosis* O^6-methylguanine methyltransferase and mutated variants. J. Bacteriol. **2013**, 195, 2728–2736.
39. Perugino, G.; Miggiano, R.; Serpe, M.; Vettone, A.; Valenti, A.; Lahiri, S.; Rossi, F.; Rossi, M.; Rizzi, M.; Ciaramella, M. Structure-function relationships governing activity and stability of a DNA alkylation damage repair thermostable protein. Nuc. Ac. Res. **2015**, 43, 8801–8816.
40. Donini, S.; Ferraris, D.M.; Miggiano, R.; Massarotti, A.; Rizzi, M. Structural investigations on orotate phosphoribosyltransferase from *Mycobacterium tuberculosis*, a key enzyme of the *de novo* pyrimidine biosynthesis. Sci. Rep. **2017**, 7, 1180.
41. Donini, S.; Garavaglia, S.; Ferraris, D.M.; Miggiano, R.; Mori, S.; Shibayama, K.; Rizzi, M. Biochemical and structural investigations on phosphorribosylpyrophosphate synthetase from *Mycobacterium smegmatis*. PLoS One **2017**, 12, 4, e0175815.
42. Lahiri, S.; Rizzi, M.; Rossi, F.; Miggiano, R.; *Mycobacterium tuberculosis* UvrB forms dimers in solution and interacts with UvrA in the absence of ligands. Proteins **2018**, 86, 98–109.
43. Presta, L.G.; Rose, G.D. Helix signals in proteins. Science **1988**, 240, 1632–1641.
44. Richardson, J.S.; Richardson D.C. Amino acid preferences for specific locations at the ends of α-helices. Science **1988**, 240, 1648–1652.
45. Koscielska-Kasprzak, K.; Cierpicki, T.; Otlewski, J. Importance of alpha-helix N-capping motif in stabilization of betabetaalpha fold. Protein Sci. **2003**, 12, 1283–1289.
46. Chan, M.K.; Mukund, S.; Kletzin, A.; Adams, M.W.W.; Rees, D.C. Structure of a hyperthermo-philic tungstopterin enzyme, aldehyde ferredoxin oxidoreductase. Science **1995**, 267, 1463–1469.
47. Miggiano, R.; Perugino, G.; Ciaramella, M.; Serpe, M.; Rejman, D.; Páv, O.; Pohl, R.; Garavaglia, S.; Lahiri, S.; Rizzi, M. Crystal structure of *Mycobacterium tuberculosis* O^6-methylguanine-DNA methyltransferase protein clusters assembled on to damaged DNA. Biochem. J. **2016**, 473, 123–133.
48. Rice, D.W.; Yip, K.S.; Stillman, T.J.; Britton, K.L.; Fuertes, A.; Connerton, J.; Pasquo, A.; Scandura, R.; Engel, P. C. Insight into the molecular basis of thermal stability from the structure determination of *Pyrococcus furiosus* glutamate dehydrogenase. FEMS Microbial Rev. **1996**, 18, 105–117.
49. Hashimoto, H.; Inoue, T.; Nishioka, M.; Fujiwara, S.; Takagi, M.; Imanaka, T.; Kai, Y. Hyperthermostable protein structure maintained by intra and inter-helix ion-pairs in archaeal O^6-methylguanine-DNA methyltransferase. J. Mol. Biol. **1999**, 292, 707–716.
50. Moore, M.H.; Gulbis, J.M.; Dodson, E.J.; Demple, B.; Moody, P.C. Crystal structure of a suicidal DNA repair protein: The Ada O^6-methylguanineDNA methyltransferase from *E. coli*. EMBO J. **1994**, 13, 1495–1501.
51. Roberts, A.; Pelton, J.G.; Wemmer, D.E. Structural studies of MJ1529, an O^6-methylguanine-DNA methyltransferase. Magn. Reson. Chem. **2006**, 44, S71–S82.

52. Perugino, G.; Vettone, A.; Illiano, G.; Valenti, A.; Ferrara, M.C.; Rossi, M.; Ciaramella, M. Activity and regulation of archaeal DNA alkyltransferase: Conserved protein involved in repair of DNA alkylation damage. J. Biol. Chem. **2012**, 287, 4222–4231.
53. Olsson, M.; Lindahl, T. Repair of alkylated DNA in *Escherichia coli*. Methyl group transfer from O^6-methylguanine to a protein cysteine residue. J. Biol. Chem. **1980**, 255, 10569–10571.
54. Wu, R.S.; Hurst-Calderone, S.; Kohn K.W. Measurement of O^6-alkylguanine-DNA-alkyltransferase activity in human cells and tumor tissues by restriction endonuclease inhibition. Cancer Res. **1987**, 47, 6229–6235.
55. Klein, S.; Oesch, F. Assay for O^6-alkylguanine-DNA-alkyltransferase using oligonucleotides containing O^6-methylguanine in a BamHI recognition site as substrate. Anal. Biochem. **1992**, 205, 294–299.
56. Luu, K.X.; Kanugula, S.; Pegg, A.E.; Pauly, G.T.; Moschel, R.C. Repair of oligodeoxyribonucleotides by O(6)-alkylguanine-DNA alkyltransferase. Biochemistry **2002**, 41, 8689–8697.
57. Tintoré, M.; Aviñó, A.; Ruiz, F.M.; Eritja, R.; Fábrega, C. Development of a novel fluorescence assay based on the use of the thrombin binding aptamer for the detection of O^6-alkyl-guanine-DNA alkyltransferase activity. J. Nuc. Ac. **2010**, 2010, 632041.
58. Vettone, A.; Serpe, M.; Hidalgo, A.; Berenguer, J.; del Monaco, G.; Valenti, A.; Ciaramella, M.; Perugino, G. A novel thermostable protein-tag: optimization of the *Sulfolobus solfataricus* DNA-alkyl-transferase by protein engineering. Extremophiles **2016**, 20, 1–13.
59. Morrone, C.; Miggiano, R.; Serpe, M.; Massarotti, A.; Valenti, A.; Del Monaco, G.; Rossi, M.; Rossi, F.; Rizzi, M.; Perugino, G. Interdomain interactions rearrangements control the reaction steps of a thermostable DNA alkyltransferase. Biochim. Biophys. Acta **2017**, 1861, 86–96.
60. Elder, R.H.; Margison, G.P.; Rafferty, J.A. Differential inactivation of mammalian and *Escherichia coli* O^6-alkylguanine-DNA alkyltransferases by O^6-benzylguanine. Biochem. J. **1994**, 298, 231–235.
61. Goodtzova, K.; Kanugula, S.; Edara, S.; Pauly, G.T.; Moschel, R.C.; Pegg, A.E. Repair of O^6-benzylguanine by the *Escherichia coli* Ada and Ogt and the human O^6-alkylguanine-DNA alkyltransferase. J. Biol. Chem. **1997**, 272, 8332–8339.
62. Miggiano, R.; Valenti, A.; Rossi, F.; Rizzi, M.; Perugino, G.; Ciaramella, M. Every OGT Is Illuminated ... by Fluorescent and Synchrotron Lights. Int. J. Mol. Sci. **2017**, 18, 2613–2630.
63. Melikishvili, M.; Rasimas, J.J.; Pegg, A.E.; Fried, M.G. Interactions of human O(6)-alkylguanine-DNA alkyltransferase (AGT) with short double-stranded DNAs. Biochemistry **2008**, 47, 13754–13763.
64. Brunk, E.; Mollwitz, B.; Rothlisberger, U. Mechanism to Trigger Unfolding in O^6-Alkylguanine-DNA Alkyltransferase. ChemBioChem **2013**, 14, 703–710.
65. Gautier, A.; Juillerat, A.; Heinis, C.; Corrêa, I.R., Jr.; Kindermann, M.; Beaufils, F.; Johnsson, K. An engineered protein-tag for multi-protein labeling in living cells. Chem. Biol. **2008**, 15, 128–136.
66. Rossi, F.; Morrone, C.; Massarotti, A.; Ferraris, D.M.; Valenti, A.; Perugino, G.; Miggiano, R. Crystal structure of a thermophilic O^6-alkylguanine-DNA alkyltransferase-derived self-labeling protein-tag in covalent complex with a fluorescent probe. Biochem. Biophys. Res. Comm. **2018**, 500, 698–703.
67. Cava, F.; de Pedro M.A.; Blas-Galindo, E.; Waldo, G.S.; Westblade, L.F.; Berenguer, J. Expression and use of superfolder green fluorescent protein at high temperatures *in vivo*: a tool to study extreme thermophile biology. Environ. Microbiol. **2008**, 10, 605–613.
68. Keppler, A.; Pick, H.; Arrivoli, C.; Vogel, H.; Johnsson, K. Labeling of fusion proteins with synthetic fluorophores in live cells. Proc. Natl. Acad. Sci. **2004**, 10, 9955–9959.
69. Valenti, A.; Perugino, G.; D'Amaro, A.; Cacace, A.; Napoli, A.; Rossi, M.; Ciaramella, M. Dissection of reverse gyrase activities: Insight into the evolution of a thermostable molecular machine. Nucleic Acids Res. **2008**, 36, 4587–4597.
70. Valenti, A.; Perugino, G.; Nohmi, T.; Rossi, M.; Ciaramella, M. Inhibition of translesion DNA polymerase by archaeal reverse gyrase. Nuc. Ac. Res. **2009**, 37, 4287–4295.
71. Perugino, G.; Valenti, A.; D'Amaro, A.; Rossi, M.; Ciaramella, M. Reverse gyrase and genome stability in hyperthermophilic organisms. Biochem. Soc. Trans. **2009**, 37, 69–73.
72. Valenti, A.; Perugino, G.; Rossi, M.; Ciaramella, M. Positive supercoiling in thermophiles and mesophiles: of the good and evil. Biochem. Soc. Trans. **2011**, 39, 58–63.
73. Visone, V.; Han, W.; Perugino, G.; Del Monaco, G.; She, Q.; Rossi, M.; Valenti, A.; Ciaramella, M. *In vivo* and *in vitro* protein imaging in thermophilic archaea by exploiting a novel protein tag. PLoS ONE **2017**, 10, e0185791.

74. Morita, R.; Nakagawa, N.; Kuramitsu, S.; Masui, R. An *O*^{6}-methylguanine-DNA methyltransferase-like protein from *Thermus thermophilus* interacts with a nucleotide excision repair protein. J. Biochem. **2008**, 144, 267–277.
75. Schärer, O.D. Alkyltransferase-like Proteins: Brokers Dealing with Alkylated DNA Bases. Mol. Cell **2012**, 47, 3–4.
76. Tubbs, J.L.; Latypov, V.; Kanugula, S.; Butt, A.; Melikishvili, M.; Kraehenbuehl, R.; Fleck, O.; Marriott, A.; Watson, A.J.; Verbeek, B.; McGown, G.; Thorncroft, M.; Santibanez-Koref, M.F.; Millington, C.; Arvai, A.S.; Kroeger, M.D.; Peterson, L.A.; Williams D.M.; Fried, M.G.; Margison, G.P.; Pegg, A.E.; Tainer, J.A. Flipping of alkylated DNA damage bridges base and nucleotide excision repair. Nature **2009**, 459, 808–813.
77. Latypov, V.F.; Tubbs, J.L.; Watson, A.J.; Marriott, A.S.; McGown, G.; Thorncroft, M.; Wilkinson, O.J.; Senthong, P.; Butt, A.; Arvai, A.S.; Millington, C.L.; Povey, A.C.; Williams, D.M.; Santibanez-Koref, M.F.; Tainer, J.A.; Margison, G.P.; Adams, C.A.; Fried, M.G. Atl1 regulates choice between global genome and transcription-coupled repair of O(6)-alkylguanines. Mol Cell. **2012**, 47, 50–60.
78. Zhou Z.; Hartmann, M. Progress in enzyme immobilization in ordered mesoporous materials and related applications. Chem Soc Rev **2013**, 42, 3894–3912.
79. Tosa T.; Mori T.; Fuse N.; Chibata I. Studies on continuous enzyme reactions. I. Screening of carriers for preparation of water-insoluble aminoacylase. Enzymologia **1966**, 31, 214–224.
80. Mohamad, N.R.; Che Marzuki, N.H.; Buang, N.A.; Huyop, F.; Wahab, R.A. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotech. Biotech. Equip. **2015**, 29, 205–220.
81. Nguyen, H.H.; Kima, M. An Overview of Techniques in Enzyme Immobilization. App. Sci. Conv. Tech. **2017**, 26, 157–163.
82. Jakub Zdarta, J.; Meyer, A.S.; Jesionowski, T.; Pinelo, M. A General Overview of Support Materials for Enzyme Immobilization: Characteristics, Properties, Practical Utility. Catalysts **2018**, 8, 92.
83. Sirisha, V.L.; Jain, A.; Jain, A. Chapter Nine - Enzyme Immobilization: An Overview on Methods, Support Material, and Applications of Immobilized Enzymes. Adv. Food Nut. Res. **2016**, 79, 179–211.
84. Gurian-Sherman, D.; Lindow, S.E. Bacterial ice nucleation: significance and molecular basis. FASEB J. **1993**, 7, 1338–1343.
85. Cochet, N.; Widehem, P. Ice crystallization by *Pseudomonas syringae*. Appl. Microbiol. Biotechnol. **2000**, 54, 153–161.
86. Merlo, R.; Del Prete, S.; Valenti, A.; Mattossovich, R.; Carginale, V.; Supuran, C.T.; Capasso, C.; Perugino, P. An AGT-based protein-tag system for the labelling and surface immobilization of enzymes on *E. coli* outer membrane. J. Enz. Inhib. Med. Chem. **2019**, 34, 490–499.
87. Del Prete, S.; Merlo, R.; Valenti, A.; Mattossovich, R.; Rossi, M.; Carginale, M.;, Supuran, C.T.; Perugino, G.; Capasso, C. Thermostability enhancement of the α-carbonic anhydrase from *Sulfurihydrogenibium yellowstonense* by using the anchoring-and-selflabelling-protein-tag system (ASL^{tag}). J. Enz. Inhib. Med. Chem. **2019**, 34, 946–954.
88. Del Prete, S.; Perfetto, R.; Rossi, M.; Alasmary, F.A.S.; Osman, S.M.; AlOthman, Z.; Supuran, C.T.; Capasso, C. A one-step procedure for immobilising the thermostable carbonic anhydrase (*Ss*pCA) on the surface membrane of *Escherichia coli*. J. Enz. Inhib. Med. Chem. **2017**, 32, 1120–1128.
89. Mattossovich, R.; Merlo R.; Fontana, A.; d'Ippolito, G.; Terns, M.P; Watts, E.A.; Valenti, A.; Perugino, P. A journey down to hell: new thermostable protein-tags for biotechnology at high temperatures. Extremophiles **2020**, 24, 81–91.
90. Fiala, G.; Stetter, K.O. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100 °C. Arch. Microb. **1986**, 145, 56–61.
91. Lundberg, K.S.; Shoemaker, D.D.; Adams, M.W.W.; Short, J.M.; Sorge, J.A.; Mathur, E.J. High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. Gene **1991**, 108, 1–6.
92. Nishikori, S.; Shiraki, K.; Yokota, K.; Izumikawa, N.; Fujiwara, S.; Hashimoto, H.; Imanaka, T.; Takagi, M. Mutational effects on *O*^{6}-methylguanine-DNA methyltransferase from hyperthermophile: contribution of ion-pair network to protein thermostability. J. Biochem. **2004**, 135, 525–532.
93. Niesen F.H.; Berglund, H.; Vedadi, M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nat Protoc **2007**, 2, 2212–2221.

94. Belkin, S.; Wirsen, C.O.; Jannasch, H.W. A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. App. Environ. Microbiol. **1986**, 51, 1180–1185.
95. Jannasch, H.W.; Huber, R.; Belkin, S.; Stetter, K.O. *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus Thermotoga. Arch. Microbiol. **1988**, 150, 103–104.
96. Conners, S.B.; Mongodin, E.F.; Johnson, M.R.; Montero, C.I.; Nelson, K.E.; Kelly, R.M. Microbial biochemistry, physiology, and biotechnology of hyperthermophilic Thermotoga species. FEMS Microb. Rev. **2006**, 30, 872–905.
97. Zhang, J.; Shi, H.; Xu, L.; Zhu, X.; Li, X. Site-directed mutagenesis of a hyperthermophilic endoglucanase Cel12B from *Thermotoga maritima* based on rational design. PLoS ONE **2015**. doi. org/10.1371/journ al.pone.01338 24
98. Fink, M.; Trunk, S.; Hall, M.; Schwab, H.; Steiner, K. Engineering of TM1459 from *Thermotoga maritima* for increased oxidative alkene cleavage activity. Front. Microb. **2016**, 7, 1–9.
99. Donaldson, T.; Iozzino, L.; Deacon, L.J.; Billones, H.; Ausili, A.; D'Auria, S.; Dattelbaum, J.D. Engineering a switch-based biosensor for arginine using a *Thermotoga maritima* periplasmic binding protein. An. Biochem. **2017**, 525, 60–66.
100. Han, D.; Xu, Z. Development of a pyrE-based selective system for Thermotoga sp. strain RQ7. Extremophiles **2017**, 21, 297–306.
101. Pegg, A.E. Repair of O^6-alkylguanine by alkyltransferases. Mutat. Res. **2000**, 462, 83–100.
102. Margison, G.P.; Povey, A.C.; Kaina, B.; Santibanez Koref, M.F. Variability and regulation of O^6-alkylguanine-DNA alkyltransferase. Carcinogenesis **2003**, 24, 625–635.
103. Mollwitz, B.; Brunk, E.; Schmitt, S.; Pojer, F.; Bannwarth, M.; Schiltz, M.; Rothlisberger, U.; Johnsson, K. Directed evolution of the suicide protein O^6-alkylguanine-DNA alkyltransferase for increased reactivity results in an alkylated protein with exceptional stability. Biochemistry **2012**, 51, 986–994.
104. Hale, C.R.; Zhao, P.; Olson, S.; Duff, M.O.; Graveley, B.R.; Wells, L.; Terns, R.M.; Terns, M.P. RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. Cell **2009**, 139, 945–956.
105. Terns, R.M.; Terns, M.P. The RNA- and DNA-targeting CRISP-Cas immune systems of *Pyrococcus furiosus*. Biochem. Soc. Trans. **2013**, 41, 1416–1421.

© 2020 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).