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Review Article

DNA Damage Induced by Alkylating Agents and Repair Pathways

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The cytotoxic effects of alkylating agents are strongly attenuated by cellular DNA repair processes, necessitating a clear understanding of the repair mechanisms. Simple methylating agents form adducts at *N*- and *O*-atoms. *N*-methylations are removed by base excision repair, AlkB homologues, or nucleotide excision repair (NER). O^6-methylguanine (MeG), which can eventually become cytotoxic and mutagenic, is repaired by O^6-methylguanine-DNA methyltransferase, and O^6MeG:T mispairs are recognized by the mismatch repair system (MMR). MMR cannot repair the O^6MeG/T mispairs, which eventually lead to double-strand breaks. Bifunctional alkylating agents form interstrand cross-links (ICLs) which are more complex and highly cytotoxic. ICLs are repaired by complex of NER factors (e.g., endnuclease xeroderma pigmentosum complementation group F-excision repair cross-complementing rodent repair deficiency complementation group 1), Fanconi anemia repair, and homologous recombination. A detailed understanding of how cells cope with DNA damage caused by alkylating agents is therefore potentially useful in clinical medicine.

1. Introduction

Alkylating drugs are the oldest class of anticancer drugs still commonly used; they play an important role in the treatment of several types of cancers [1]. Most alkylating drugs are monofunctional methylating agents (e.g., temozolomide [TMZ], *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine [MNNG], and dacarbazine), bifunctional alkylating agents such as nitrogen mustards (e.g., chlorambucil and cyclophosphamide), or chloroethylating agents (e.g., nimustine [ACNU], carmustine [BCNU], lomustine [CCNU], and fotemustine).

Simple methylating agents form adducts at the *N*- and *O*-atoms in DNA bases. *N*-methylation adducts comprise more than 80% of methylated bases. These alkyl DNA base adducts exhibit different stabilities. For example, N^7-methylguanine (N^7MeG) is the most stable *N*-methylation adduct in vitro with a half-life ($t_{1/2}$) no longer than 80 h [2]. Although O^6-methylguanine (O^6MeG) accounts for only 0.3% (for methyl methanesulfonate) to 8% (for methylnitrosourea) of the total DNA methyl adducts, it is stable and persists in the absence of the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT) [3–5]. O^4-methylthymine (O^4MeT) is produced at a much lower level (<0.4%) [2], and its mutagenicity and cytotoxicity are unclear. In general, *O*-alkylations (e.g., O^6alkylG and O^4alkylT) are highly mutagenic and genotoxic, whereas *N*-alkylations (e.g., N^3alkylA and N^1alkylA) are cytotoxic, but less mutagenic [6–9]. The primary products of methylating agents, *N*-alkylated purines, are efficiently removed by base excision repair (BER) or human AlkB homologues (hABH). BER repairs N^7MeG, N^3MeA, and N^3MeG, whereas hABH repairs N^1MeA, N^3MeC, N^3MeT, and N^1MeG [10].

One-step repair of O^6MeG involves transferring the alkyl group from the oxygen in the guanine to a cysteine residue in the catalytic pocket of MGMT [10]. Nucleotide excision repair (NER) is an elaborate repair system that removes bulky lesions from DNA in 27-nt to 29-nt oligomers. Because it is also capable of removing nonbulky lesions such as apurinic/apyrimidinic sites and O^6MeG residues, NER plays

a backup role for other repair systems [11]. Mismatch repair (MMR) is also important in the repair of O^6MeG. If it is left unrepaired, replication over the O^6MeG results in an O^6MeG:T mismatch or O^6MeG:C ambiguous pair [12]. In the next round of replication, the O^6MeG:T becomes an A:T transition mutation, or the O^6MeG:C is replicated again as an O^6MeG:C pair or becomes an O^6MeG:T mismatch [13]. The O^6MeG:T or O^6MeG:C is recognized by the MutSα complex (hMSH2 and hMSH6), which initiates MMR to create a gapped duplex by incision of the newly replicated strand [13]. If O^6MeG remains in the template, a futile repair loop can eventually result in highly toxic double-strand breaks (DSBs), which are intermediates in apoptotic and DSB repair pathways [13]. Accordingly, DSB repair pathways are activated by methylating agents [14, 15].

Bifunctional alkylating agents, such as chlorambucil or BCNU, are commonly used anticancer drugs. DNA lesions produced by these agents require complex repair mechanisms. The primary chloroethyl adducts at O^6G are repaired by MGMT, but the secondary interstrand cross-links (ICLs) require NER factors (e.g., endnuclease xeroderma pigmentosum complementation group F-excision repair cross-complementing rodent repair deficiency complementation group 1 (XPF-ERCC1)) for incision, Fanconi anemia (FA) repair, and homologous recombination (HR) for complete repair [16].

This paper will focus on the repair pathways for O^6MeG generated by methylating agents and those for ICLs generated by bifunctional alkylating agents. We will also briefly discuss other alkylation damage defense and processing functions (hABH and BER).

2. DNA Repair Mechanisms for DNA Damage Induced by Methylating Agents

2.1. MGMT (Figure 1(a)). MGMT repairs O^6-alkylation adducts but irreversibly inactivates MGMT itself in the process. In the absence of active MGMT, O^6MeG forms O^6MeG/T mismatches during replication. Early studies demonstrated that MGMT-deficient cells unable to repair O^6MeG damage were more sensitive to the effects of methylating agents than normal cells expressing MGMT [17]. This observation has been utilized experimentally and clinically to target cells with an MGMT inhibitor, the O^6MeG analogue O^6benzylG [18]. However, in some tumors, *p*53 dysfunction suppresses MGMT expression [19, 20] or hypermethylation of the MGMT promoter results in gene silencing [21]. The low basal MGMT activity makes these cells less vulnerable to the effects of O^6benzylG. Kaina et al. reported that about 5% of all solid tumors assayed in their laboratory were completely deficient in MGMT [10]. In particular, 17% to 30% of gliomas lack MGMT [22, 23]. Because drug efficacy is likely to be limited if only MGMT is targeted in these tumors, new molecular targets are being sought.

2.2. MMR (Figure 1(b)). The cytotoxicity of monofunctional alkylating agents requires a functional MMR in the target cells. In fact, mammalian cells proficient in MMR are generally about 100-fold more sensitive to alkylating agents than their MMR-deficient counterparts [24, 25]. In MMR-deficient cells, DNA damage accumulates but does not trigger cell death. Thus, resistance to these cytotoxic agents is associated with loss of MMR activity, particularly in the absence of MGMT [26, 27]. The mechanism of action of monofunctional alkylating agents has been studied in cell lines and mouse models; results indicate that replication over unrepaired O^6MeG:C results in an O^6MeG:T mismatch (or possibly an O^6MeG:C ambiguous pair). In the next round of replication, an O^6MeG:T mismatch becomes an A:T transition mutation. An O^6MeG:T or O^6MeG:C pair is recognized by the MutSα complex, which initiates MMR. MMR creates a gapped duplex after incision of the newly replicated strand. The mere presence of MeG in the genomic DNA of MMR-proficient cells is not cytotoxic, even if the cells are allowed to undergo a round of replication during which MeG:C and MeG:T pairs form. To activate the G_2/M DNA damage checkpoint, these mispairs must be recognized and processed. Cells treated with MNNG are not arrested in the first G_2/M checkpoint, but G_2/M arrest is commonly observed in the second cell cycle [28].

2.3. DSB Repair (Figure 1(c)). Although alkylating agents do not directly induce DSBs, DSBs are detected in wild-type cells and other cell culture systems after the processing of DNA lesions induced by alkylating agents [14, 15, 29, 30]. DSBs lead to cell death; therefore, cells defective in DSB repair are thought to be more sensitive to alkylating agents. Consistent with this hypothesis, studies have reported that DSB repair pathways are involved in the repair of DNA damage induced by alkylating agents [14, 15, 29].

DSBs are repaired through the HR and nonhomologous end joining (NHEJ) pathways [31]. In human cells, HR proteins include members of the MRN complex, which consist of meiotic recombination 11 (MRE11)/radiation-sensitive mutant 50 (Rad50)/Nijmegen breakage syndrome 1 (NBS1) as well as Rad51, the Rad51 paralogs (Rad51B, Rad51C, Rad51D, X-ray repair cross-complementing group 2 (XRCC2), and XRCC3), Rad54, and Rad54B [31]. Proteins involved in the NHEJ pathway include Ku70/80, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ligase IV (Lig4), XRCC4 and Artemis [31].

HR, which is a generally error-free pathway, uses DNA homology to direct DNA repair; an undamaged chromatid serves as template for the repair of a broken sister chromatid. The products of the breast cancer susceptibility genes, BRCA1 and BRCA2 (also known as FA complementation group D1 or FANCD1), are also involved in the HR pathway [32].

HR is required for MNNG-treated cells to transition into the second cell cycle. Most mammalian cells that undergo cell cycle arrest after the second S phase die; however, the surviving cells show a high frequency of sister chromatid exchanges (SCEs), indicative of DSB repair at collapsed replication forks [33]. Roos et al. reported that BRCA2/XRCC2-dependent HR, but not NHEJ, protects against O^6MeG-triggered DSBs and chromosomal aberrations, leading to SCEs [14].

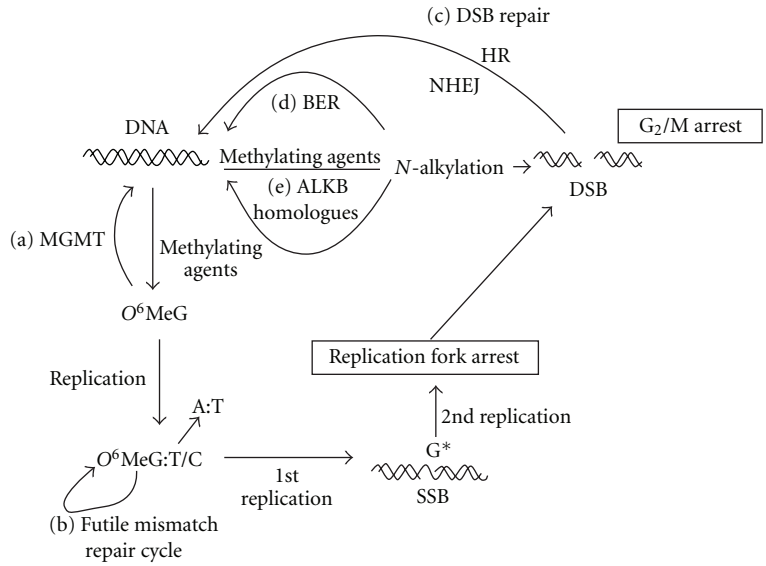

Figure 1: Pathways for DNA damage induced by methylating agents. (a) O^6-methylguanine-DNA methyltransferase (MGMT) removes the methyl adduct from O^6MeG in one step. If left unrepaired, O^6MeG:C ambiguous pairs or O^6MeG:T mismatch pairs can form during replication. In the next round of replication, O^6MeG:T pairs can become A:T transition mutations. (b) O^6MeG:T and O^6MeG:C pairs are recognized by the mismatch repair (MMR) system, which creates a single-strand break (SSB), cause replication arrest, and finally leads to a double-strand break (DSB). O^6MeG:T/C does not induce cell cycle arrest at the first G_2/M DNA damage checkpoint, but G_2/M arrest is commonly observed in the second cell cycle. (c) Homologous recombination (HR) and nonhomologous end joining (NHEJ) may play a role in the repair of DSBs. *N*-alkylations are repaired by either (d) base excision repair (BER), or (e) AlkB homologues, and if not repaired, DSBs occur.

NHEJ, which is the simplest way to repair a DSB, involves the religation of broken DNA ends without a template; this type of repair does not preserve the original genetic information. NHEJ eliminates DSBs during the G_1 phase of the cell cycle, when the lack of sister chromatids prevents HR [34].

Results of a clonogenic survival study showed that Lig4 plays a more important role in the repair of TMZ-induced DSBs than XRCC2 or Rad54 [15]. DSBs, which may arise from adducts other than O^6MeG, such as TMZ-induced *N*-methylpurines, are repaired within 24 h in *Lig4*-proficient cells. In contrast, up to 80% of the DSBs in $Lig4^{-/-}$ cells were not repaired [15]. In A172 glioblastoma cells, siRNA silencing of *Lig4* increased cellular sensitivity to TMZ. After TMZ treatments, A172 cells with silenced *Lig4* exhibited a 62.5% decrease in survival compared with control A172 cells; thus, modulating Lig4 activity may enhance tumor sensitivity to TMZ [15].

2.4. BER (Figure 1(d)). The alkylation adducts N^7MeG, N^3MeA, and N^3MeG are repaired by the BER system, the main DNA repair system in mammalian cells used to eliminate small DNA base lesions [16]. Damaged bases are removed by a lesion-specific DNA glycosylase, in this case alkyladenine DNA glycosylase (Aag). The resulting abasic site is recognized by an apurinic/apyrimidinic endonuclease, APE1, which incises the damaged strand, leaving 3′-OH and 5′deoxyribose phosphate (5′-dRP) groups at the margins. A DNA polymerase β- (pol-β-)mediated DNA synthesis step fills the single nucleotide gap [35, 36] and removes the cytotoxic 5′-dRP group [37, 38]. Alternatively, DNA polymerase λ (pol-λ) or DNA polymerase-ι (pol-ι), both of which possess 5′-dRP lyase activity, may participate in BER to remove this toxic repair intermediate [39–41]. Finally, DNA ligase I, or a complex of DNA ligase III and XRCC1, conducts the final, nick-sealing step in the pathway [42].

In the absence of pol-β, cells cannot repair the BER intermediate 5′dRP and are thus hypersensitive to the alkylating agent methyl methanesulfonate [37, 38, 43, 44]. For example, fibroblasts from a pol-β-null mutant mouse are highly sensitive to monofunctional alkylating agents, but not to BCNU [45]. Similarly, RNA interference-mediated pol-β suppression boosts TMZ efficacy, although a deficiency in pol-ι or pol-λ does not increase TMZ-mediated cytotoxicity [46]. Furthermore, loss of pol-β coupled with TMZ treatment triggers H2AX phosphorylation, indicating activation of the DNA damage response pathway by unrepaired lesions [46]. H2AX is a histone protein that is rapidly phosphorylated on Ser139 (γH2AX) when DNA breaks are introduced in mammalian cells following external damage and replication fork collapse [47, 48]. Poly(ADP-ribose) polymerase-1 (PARP-1) is activated by strand breaks and participates in gap sealing with DNA ligase III and XRCC1, but deficiencies in the subsequent steps of BER increase sensitivity to alkylating agents. Inhibition of PARP-1 by the inhibitor AG14361 restores sensitivity to TMZ in MMR-deficient cells that have lost killing sensitivity to O^6MeG *via* the MGMT/MMR pathway [49]. The combination of TMZ with PARP-1 inhibitors is currently under investigation in several Phase I-II clinical trials.

2.5. Direct Reversal of Alkylation Damage by AlkB Homologues (Figure 1(e)). The *E. coli* protein AlkB is an oxidative DNA demethylase that repairs the cytotoxic lesions N^1MeA and N^3MeC. A detailed mapping of the human genome has identified eight hABH homologues. ABH2 and ABH3 belong to the alpha-ketoglutarate- and Fe(II)-dependent dioxygenase superfamily. These proteins repair N^1MeA, N^3MeC, N^3MeT, and N^1MeG by oxidative demethylation [50, 51]. Although hABH2 preferentially repairs double-stranded DNA, hABH3 acts more efficiently on single-stranded nucleic acids. Accordingly, hABH2 relocates to replication foci during S-phase, which suggested that hABH2 repairs DNA close to replication forks, whereas hABH3 maintains nuclear single-stranded DNA and RNA, potentially targeting genes undergoing transcription.

3. Repair of Cross-links Induced by Bifunctional Alkylating Agents

Bifunctional alkylating agents (e.g., nitrogen mustards (melphalan, chlorambucil, cyclophosphamide, and ifosfamide) and chloroethylnitrosoureas (BCNU and CCNU)) possess two reactive sites. These agents cross-link DNA with proteins or, alternatively, cross-link two DNA bases within the same DNA strand (intrastrand cross-links) or on opposite DNA strands (ICLs). ICLs, which block replication forks, are the most serious cytotoxic lesions produced by most bifunctional drugs. Accordingly, the extent of ICLs correlates well with the cytotoxicity of nitrogen mustard drugs [52].

Nitrogen mustards form N^7G:N^7G cross-links, and chloroethylnitrosoureas form N^1G:N^3C cross-links [53]. The chloroethylated O^6G of the N^1G:N^3C cross-link can be repaired by MGMT; however, this adduct is unstable and undergoes intramolecular rearrangement producing an intermediary N^1-O^6-ethanoG. The N^1-O^6-ethanoG adduct may react with cytosine in the complementary strand to yield a highly toxic ICL between position 1 in the guanine residue and position 3 in the cytosine residue (1-(3-cytosinyl)-2-(1-guanosinyl)-ethane) [53].

ICL repair mechanisms are complex; therefore, they are only briefly summarized here. An ICL represents a formidable block to the DNA replication machinery and is unique in requiring a combination of FA repair, NER, translesion synthesis (TLS), and HR repair for efficient repair [54]. Although the FA pathway was initially characterized in terms of DNA cross-link repair, this pathway is also involved in homologous recombination and resolution of the replication arrest [55, 56]. Thirteen FA genes have been identified [54], although the precise function of many of these FA proteins is unclear. The FA core complex, which consists of eight FA proteins, is activated by DNA damage. Specifically, the FA proteins FANCM and FANCA Associating Polypeptide 24 form a heterodimer that binds DNA [57, 58] and appears to be involved in sensing DNA replication forks blocked at cross-links. The NER proteins ERCC1 and XPF make incisions on either side of the cross-link to generate a gap. The gap is then filled by translesion synthesis (TLS) polymerases ζ (Rev3 and Rev7 subunits) and Rev1 (part of the Rev3-Rev7 complex [59]). The FA core complex monoubiquitinates FANCD2 and its paralog FANCI, and the ubiquitinated FANCD2 then interacts with FANCD1 to promote HR [54].

Incision at the ICL could occur before or after lesion bypass, leaving a DSB subject to HR or NHEJ [60]. As expected, XRCC2 and Rad54 are involved in the repair of ACNU-induced DSBs, but surprisingly Lig4 plays the most important role in this process [29]. In Lig4$^{-/-}$ cells, levels of phosphorylated histone γH2AX increased more than 4-fold at 12 h and 6-fold at 24 h after ACNU treatment compared to its initial levels. In contrast, γH2AX levels were not markedly altered by ACNU in normal cells. In addition, ACNU treatment markedly reduced the colony-forming ability of A172 glioblastoma cells transfected with siRNA against Lig4 or XRCC2 compared to controls [29]. However, *Lig4* siRNA rendered cells more sensitive to the effects of ACNU than did *XRCC2* siRNA [29]. These data suggest NHEJ may also be involved in removing DSBs formed by unrepaired ICLs.

4. Conclusion

DNA repair pathways attenuate the therapeutic effects of alkylating agents; therefore, characterization of the repair pathways is essential for developing new treatments. For example, MGMT promoter hypermethylation results in gene silencing and therefore decreased MGMT activity; therefore, MGMT promoter hypermethylation may be a useful way to enhance the therapeutic efficacy of TMZ [61, 62].

Currently, clinical trials are testing DNA repair inhibitors that target PARP, BER, or MGMT in combination with alkylating agents [63]. In the case of O^6benzylG, a phase I clinical trial has defined the maximum tolerated dose of a single dose of TMZ when combined with O^6benzylG and has determined the dose of O^6benzylG that depletes tumor MGMT activity for 48 h [64]. In addition, when combined with cytotoxic chemotherapy, myelosuppression appears to be significantly enhanced by O^6benzylG, significantly reducing the required doses of alkylating agents [65]. The success of such approaches will depend on selective targeting of the tumor. Locoregional chemotherapy has recently been shown to improve the survival of glioma patients [66]. Therefore, combining a locoregional delivery system with the simultaneous downregulation of DNA repair pathways may decrease the amount of alkylating agent needed for chemotherapy, thereby reducing the severe side effects. In addition, new inhibitors against specific repair proteins, such as pol-β, BRCA2, or Lig4, should be developed because resistance against currently available inhibitors may develop.

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