From old alkylating agents to new minor groove binders

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Abstract

Alkylating agents represent the oldest class of anticancer agents with the approval of mechloretamine by the FDA in 1949. Even though their clinical use is far beyond the use of new targeted therapies, they still occupy a major place in the treatment of specific malignancies, sometimes representing the unique option for the treatment of refractory tumors. Here, we are reviewing the major classes of alkylating agents, with a particular focus on the latest generations of compounds that specifically target the minor groove of the DNA. These naturally occurring derivatives have a unique mechanism of action that explains the recent regain of interest in developing new classes of alkylating agents that could be used in combination with other anticancer drugs to enhance tumor response in the clinic.

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Keywords: Alkylating agents; DNA adducts; DNA repair; O6-methylguanine; DNA crosslinks

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1. Introduction

Alkylating agents were first used as chemical weapons during the First World War. They induced severe vesicant effects that were accompanied by bone marrow aplasia and pancytopenia several days following intoxication. This fortuitous observation led to the first experimentation of mustard gas as anticancer agents to target carcinogen-induced tumors in mice [1]. In the early 30s, the first trials in men showed interesting durable responses in patients with skin cancers and sarcomas [1]. Despite the Geneva Convention in 1925, a vast research program was secretly carried out by the US Office of Scientific Research and Development in order to identify new chemical weapons. Another major trigger for the discovery of alkylating agents’ anticancer activity was the accidental spill of sulphur mustards that occurred during World War II in the Italian harbor of Bari. On December 2nd 1943, 17 ships were destroyed by a surprise raid of the German aviation, including the SS John Harvey vessel that secretly transported a cargo of more than 100 tonnes of mustard bombs. Among soldiers that were exposed, a significant rate of severe lymphoid hypoplasia and myelosuppression was observed. This led to further investigations of nitrogen mustards as potential anticancer agents by Gilman and Goodman who showed remarkable activity in the treatment of lymphomas. These results were only published in 1946 [2] but were rapidly followed by the FDA approval of the first alkylating agent, mechlorethamine (Mustargen) in March 1949, and the development of a number of new classes of compounds. Even though their clinical use is far beyond the use of new targeted therapies, they still occupy a major place in the treatment of specific malignancies and sometimes represent the unique option for the treatment of refractory tumors (Table 2). Here, we are reviewing the main classes of alkylating agents with a specific emphasis on the new generations of compounds that show a rather unique mechanism of action. Used as single agents or in combination with DNA repair inhibitors, these new derivatives represent promising therapeutic alternatives for tumors refractory to standard treatments.

2. The mechanism of DNA alkylation

Alkylating agents are electrophilic entities that react with nucleophilic moieties of DNA or proteins resulting in the covalent transfer of an alkyl group [3,4]. The cytotoxic effect of these agents is mainly due to the alkylation of DNA bases that can impair essential DNA processes such as DNA replication and/or transcription. The chemical reaction of alkylation is summarized in Fig. 1A where RX represents the alkylating agent with its alkyl group (R) and a halogen atom (X), usually chlorine, and R’H the nucleophilic target of alkylation where the hydrogen atom of hydroxyl, amine, carboxyl, or sulfhydryl groups, is substituted. (B) The two types of nucleophile substitutions (SN1 and SN2) depending on the number of steps of the reaction (Fig. 1B). SN1 reaction involves the formation of a stable carboxylation with a planar structure which can be attacked by the nucleophile on both sides with equal probability, leading to a racemic product. Conversely, SN2 reaction implies the formation of a short-lived intermediate in which the leaving group is not completely detached and the nucleophile almost linked covalently, leading to an inversion of the configuration of the asymmetric carbon [4].

3. The different targets of DNA alkylation

DNA is the main target of alkylation [3–5]. While monofunctional agents generate covalent adducts with the target molecule (Fig. 2a–c), bi-functional derivatives can form cross-links (inter-strands or intra-strand) in DNA or between DNA and proteins (Fig. 2d–h). There are preferential sites of alkylation in DNA depending on the nature of the nucleophile and of the alkylation agent [6–8]. In general base alkylation predominantly occurs on position guanine N7 and O6, adenine N1 and N3, and cytosine N3 [7,9,10]. The use of Mitomycin C and of the new classes of minor groove binders also revealed guanine N2 as a target of DNA alkylation (Fig. 3). Alkylation on other sites such as adenine N6, N7, thymine O2, N3, O4, or cytosine O2 were also observed, but to a lower extent (Fig. 3 and Table 1) [8,11]. While alkylation on O6G, N1G, N2G, or O4T results in stable DNA adducts [12], alkylation on other positions lead to chemically unstable adducts that are converted to DNA damage by opening of the base ring [8,12] (Fig. 4). That is especially the case for
the most common base alkylation at N7G position which is readily converted into abasic sites by spontaneous depurination or into 5-alkyl formamidopyrimidine (Fapy) by opening of the imidazole ring [13]. Similar observations have been reported for N3A alkylation, leading to abasic sites formation [14]. The phosphodiester bond of the DNA backbone is also a substrate of alkylation because of its accessibility and the negative charge of its oxygen atoms but its consequences in terms of cytotoxicity are not demonstrated yet [6].

The cellular consequences of base alkylation have been largely studied but are still difficult to evaluate since most if not all alkylation agents do not form a unique type of lesion but a mixture of adducts. Table 1 summarizes the main mutations that are observed following base alkylation. N-alkylation usually results in toxic effects due to a blockage of DNA replication linked to the presence of alkylated bases or to DNA damage induced by the processing of these lesions by DNA repair enzymes (Fig. 4) [8]. Conversely, O-alkylations such as O6-methylguanine (O6MeG) are mutagenic and cytotoxic. Mutagenicity is due to the formation of

Table 1

<table>
<thead>
<tr>
<th>Adducts</th>
<th>Stability</th>
<th>Mutations</th>
<th>Repair pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>N7</td>
<td>Stable</td>
<td>G → C</td>
<td>BER</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G → T</td>
<td></td>
</tr>
<tr>
<td>Guanine</td>
<td>O6</td>
<td>Stable</td>
<td>G → A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G → T</td>
<td>BER</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G → C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N1</td>
<td>Stable</td>
<td>A → T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A → T</td>
<td>BER, DR (ABH)</td>
</tr>
<tr>
<td></td>
<td>N7</td>
<td>Unstable</td>
<td>A → G</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Unstable</td>
<td>C → T</td>
</tr>
<tr>
<td></td>
<td>O2</td>
<td>Unstable</td>
<td>C → A</td>
</tr>
<tr>
<td></td>
<td>O2</td>
<td>Unstable</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Thymine</td>
<td>O4</td>
<td>Stable</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T → C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T → A</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. The different kinds of alkylation products induced by mono-functional (a–c) or bi-functional (d–h) alkylation agents. The major groove of the DNA is the main target of alkylation but N2G and N3A alkylation is also observed for minor groove binding (MGB alkylation agents).

Fig. 3. Different sites of base alkylation. The size of arrows is proportional to the frequency of alkylation, regardless of the stability of the alkylated base. (*) The N2G position is the target of minor groove alkylators.
O6MeG:T mispairing that further leads to A:T transitions following two rounds of replication [7,8,15]. Alternatively, persistence of the adducted base can occur due to the inability of the Mismatch Repair (MMR) system which recognizes O6MeG:T mismatches to correct the damage, a phenomenon referred to as MMR futile cycle (Fig. 17). This can eventually lead to cytotoxic DNA single- or double-strand breaks by an MMR-dependent mechanism that remains to be elucidated [15–17].

Proteins can also be the targets of alkylation (Fig. 2). This relies on isocyanate derivatives that are produced following treatment with nitrosoureas (Fig. 9). In physiological conditions isocyanate can react with thiol or amine groups of cellular proteins, a reaction that is referred to as carbamoylation (or carbamylation). The exact list of potential substrates as well as the biological consequences of carbamoylation is presently unknown. It is usually admitted that carbamoylation results in the inactivation of the protein activity, as it was
4.1. Fig. The formation of monoalkylation products can lead to Alkyl-N7G:T base mispairing or can be the target of a second alkylation leading to N7G–N7G inter-strand cross-links.

4.2. The oxazaphosphorines (or oxazaphorines)

These alkylating agents have been synthesized in order to enhance the stability and to reduce the toxicity of nitrogen mustards. Chemically, they are characterized by a phosphorus–nitrogen bond which prevents the direct ionization of the bis(2-chloroethyl) moiety (Fig. 5B) [3,22,23]. The major derivatives from this class that are used in the clinic are cyclophosphamide and ifosfamide (Fig. 7A). These compounds are pro-drugs that require an activation step via cytochromes (P450) in the liver [3,22,23] (Fig. 7B). The result of the oxidation of carbone 4 of the heterocycle allows the formation of the nucleophile chloroethylaziridine responsible for N7G:N7G cross-linking (Fig. 7B).

4.1. The nitrogen mustards

These alkylating agents derived from sulfur mustards and were the first to be used as chemotherapeutic agents for the treatment of leukemias and lymphomas. They share a common bis(2-chloroethyl)amino motif that leads to the formation of an aziridinium ion, the electrophilic entity responsible for the establishment of the covalent link with the nucleophilic center of the base, mainly the N7 of guanines (Fig. 6) [3,20,21]. The resulting products of mono-alkylation can lead to base mispairing that is potentially mutagenic [13] (Table 1). Alternatively, the chloro group can react with the N7 position of an adjacent guanine to form intra- or inter-strand crosslinks (Fig. 6B). Nitrogen mustards are only used sporadically in the treatment of hematologic malignancies, multiple myeloma, in ovarian cancers, or refractory prostate cancers in the case of estramustine (Table 2).

4. The main classes of “classical” alkylating agents

There are 7 classes of “standard” alkylating agents, considering that mitomycin C belongs to ethylene imines and that platinum compounds, even though they do not alkylate DNA stricte sensu but form covalent adducts with it, are traditionally included in this category [3–5]. The chemical structures of the corresponding pharmacophores are presented in Fig. 5.

In the following sections, we will review the mechanisms of action that have been described for the main derivatives of each class that are commonly used in chemotherapy, and will only mention the new derivatives with promising activity in preclinical or early clinical trials.
Table 2
The alkylating agents that are used in the clinic and their main indications.

<table>
<thead>
<tr>
<th>Class of alkylating agents</th>
<th>Drugs</th>
<th>Clinical use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen mustards</td>
<td>Melphalan</td>
<td>Hematologic tumors, Myeloma, Ovarian cancers, Solid tumors</td>
</tr>
<tr>
<td></td>
<td>Chlorambucil</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bendamustine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Estramustine</td>
<td>Prostate cancers</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>Hematologic tumors, Solid tumors, Sarcoma</td>
</tr>
<tr>
<td></td>
<td>Ifosfamide</td>
<td>Sarcoma, Solid tumors</td>
</tr>
<tr>
<td></td>
<td>Trofosfamide</td>
<td>Palliative care</td>
</tr>
<tr>
<td>Oxazaphosphorines</td>
<td>Thiotepa</td>
<td>Ovarian, Breast, Bladder cancers</td>
</tr>
<tr>
<td></td>
<td>Altretamine</td>
<td>Ovarian, Lung cancers</td>
</tr>
<tr>
<td></td>
<td>Mitomycin C</td>
<td>Gastrointestinal tumors, Breast and Bladder cancers</td>
</tr>
<tr>
<td></td>
<td>Carmustine (BCNU)</td>
<td>Lymphoma, Brain tumors, Melanoma</td>
</tr>
<tr>
<td></td>
<td>Lomustine (CCNU)</td>
<td></td>
</tr>
<tr>
<td>Ethylene imines</td>
<td>Nimustine</td>
<td>Brain tumors, Solid tumors</td>
</tr>
<tr>
<td></td>
<td>Potemustine</td>
<td>Melanoma</td>
</tr>
<tr>
<td></td>
<td>Streptozotocine</td>
<td>Pancreatic and Neuroendocrine tumors</td>
</tr>
<tr>
<td>Nitrosoureas</td>
<td>Busulfan</td>
<td>Chronic Myeloid leukemia (CML)</td>
</tr>
<tr>
<td>Alkyl alcane sulfonates</td>
<td>Dacarbazine</td>
<td>Melanoma, Lymphoma, Sarcoma</td>
</tr>
<tr>
<td>Triazenes and hydrazines</td>
<td>Temozolomide</td>
<td>Glioma</td>
</tr>
<tr>
<td></td>
<td>Procarbazine</td>
<td>Lymphoma, Glioma</td>
</tr>
<tr>
<td></td>
<td>Cisplatin</td>
<td>Solid tumors</td>
</tr>
<tr>
<td>Platinum derivatives</td>
<td>Carboplatin</td>
<td>Solid tumors</td>
</tr>
<tr>
<td></td>
<td>Oxaliplatine</td>
<td>Colorectal cancers</td>
</tr>
<tr>
<td>Tetrahydroisoquinolines</td>
<td>Trabectedine</td>
<td>Sarcoma, Ovarian cancers</td>
</tr>
</tbody>
</table>

4.3. The ethylene imines

4.3.1. The polyaziridines

These alkylating agents are characterized by the presence of one or more aziridine cycles (Figs. 5C and 8A) [3,5,26,27]. Thiotepa and altretamine are the main compounds of this class. They alkylate DNA via the same mechanism as nitrogen mustards except that the aziridine cycle is not charged and therefore less reactive. Thiotepa can form covalent links between most base nucleophiles and can also form

Fig. 7. (A) Chemical structures of oxazaphosphorines. (B) The mechanism of DNA alkylation by cyclophosphamide. Cyclophosphamide is first oxidized by cytochrome P450 leading to 4-hydroxycyclophosphamide, acrolein and a dichloro intermediate, hydrolysis of which generates chloroethylaziridine that is responsible for DNA alkylation and the formation of N7G:N7G cross-links.
inter-strand N7G:N7G cross-links [26]. Thiotepa is rarely used for the treatment of ovarian and breast cancers, and as intravesical instillation for bladder cancers, as well as altretamine for the treatment of ovarian cancers and small cell lung cancers (Table 2).

4.3.2. Mitomycin C

Mitomycin C (Fig. 8B) has two aziridine cycles and a quinone moiety that confers specific properties which probably explains why this molecule is often dissociated from other ethylene imines [3,28]. It is an antibiotic extracted from the fermentation of Streptomyces bacteria. Its alkylating mechanism is complex and has been extensively studied. Mitomycin C has two electrophilic centers that can be activated: carbon 1 and 10 (Fig. 8B). Depending on the environment, different pathways have been described leading to various type of adducts. DNA alkylation by mitomycin C requires its activation by an enzymatic reduction leading to its protonation and the opening of the aziridine cycle (Fig. 8B), further inducing the linkage of a nucleophile to its carbon 1 (Fig. 8B and C) [3,28–30]. Conversely to other alkylating agents, the nucleophile is predominantly the nitrogen 2 of guanines, which positions mitomycin C in the minor groove of the DNA [31]. The mono-alkylation product can also be reduced leading to the elimination of the carbamate moiety which results in the activation of carbon 10. Bis-alkylation of a second guanine is then possible in CpG-rich regions, resulting in intra- or inter-strand cross-links depending on whether it is located on the same strand or not, respectively (Fig. 8C) [3,28]. DNA alkylation in the major groove is also possible since N7 monoadducts involving activation of carbon 10 of mitomycin C have also been observed (Fig. 8C). Clinically, Mitomycin C is still used for the treatment of various adenocarcinomas (stomach, pancreas, colon, rectum, breast and bladder) (Table 2).

4.4. The nitrosoureas

This class of alkylating agents emerged from the observation by the NCI that MNNG (1-methyl-3-nitro-1-nitrosoguanidine) (Figs. 5D and 9A) had a marked antiproliferative effect against various cancer cell lines [3,5,32–34]. Other derivatives such as MNU (1-methyl-1-nitrosourea) or BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) were even active against xenografted brain tumors in mice, demonstrating that these molecules could cross the hematoencephalic barrier [32,35]. If MNNG and MNU are still used for research purposes as “proof of concept” derivatives, other molecules have been tried or are used in the clinic: BCNU (carmustine), CCNU (lomustine), nimustine (not marketed), fotemustine and an aminoglycosylated derivative streptozocine (Fig. 9A). These agents are mainly used for the treatment of brain tumors, melanomas, and some carcinoid tumors (Table 2). Their mechanism of action relies on the formation of diazohydroxyde in basic conditions, that in turn generates a reactive cation responsible for alkylation which takes place primarily on O6 or N7 positions of guanines (Fig. 9B) [3,33,34,36]. O6G alkylation by
carmustine is described in Fig. 9B. First, a chloroethylated adduct is generated and is followed by the formation of a N1G:N3C inter-strand cross-link [3,33,34,37] (Fig. 9B). This type of lesion contributes to the cytotoxicity of nitrosoureas to the same extent as other cross-links such as O6G:N1C or N7G:N3C. Protein carbamylation on lysine or arginine residues induced by isocyanate (Fig. 9B) could also impair the activity of key proteins involved in cell survival such as DNA repair factors [3,33,34,38].

4.5. Alkyl alane sulfonates

Busulfan is the only compound of this family (Fig. 5E) to be used for the treatment of refractory chronic myeloid leukemias (Table 2) and prior to hematopoietic stem cell transplant in grafts [3,5,39]. Busulfan is a sulfonated bifunctional alkylating agent inducing N7G:N3A intra- or N7G:N7G inter-strand DNA cross-links or protein-DNA cross-links responsible for its cytotoxicity (Fig. 10) [39,40].

4.6. Triazenes and hydrazines

Triazenes are characterized by the presence of 3 adjacent nitrogen atoms (Fig. 5F) [3,5,41]. Two derivatives are used in the clinic, alone or in combination with other cytotoxic agents: dacarbazine for the treatment of melanomas, lymphomas and sarcomas, and temozolomide for the treatment of brain tumors (gliomas, glioblastomas and astrocytomas) (Table 2). Both derivatives are precursors of 5-(3-methyl-1-triazanyl)imidazole-4-carboxamide (MITC) and its byproduct the methyldiazonium ion responsible for alkylation [3,5,41,42] (Fig. 11A). DNA methylation by dacarbazine and temozolomide occurs on adenine N3 and guanine N7 and O6, the latter being responsible for the major part of their cytotoxicity (Fig. 11A). O6-methyl guanine is mutagenic due to its mispairing with thymine and subsequent G:C to A:T transitions (Table 1), the persistence of which leads to cytotoxic effects by the generation of secondary strand breaks [3,5,41,42].

Procarbazine belongs to the hydrazines family [3,5,43]. It was first developed as a mono-amine oxidase inhibitor but was rapidly used for its anticancer property linked to its methylhydrazine moiety. Procarbazine is also a prodrug that generates the highly reactive diazonium ion by enzymatic conversion [43–45] (Fig. 11B). As many monofunctional agents, procarbazine induces O6G and N7G mono-adducts and subsequent DNA breaks that are responsible for its cytotoxicity [46]. It is still used in drug combinations for the treatment of lymphomas and brain tumors.

4.7. Platinum derivatives

Platinum derivatives (Fig. 5G) have emerged in the clinic in the late 70s with the use of cisplatin (cis diamino-dichloroplatinum, CDDP) and its spectacular results in testicular cancers despite a high renal toxicity [5,47]. This led to the synthesis of the less toxic derivative carboplatin and a third generation of compounds called DACH (diaminocyclohexyl)-platin among which oxaliplatin and tetratplatin (Fig. 12A). Besides tetratplatin that was not marketed, platinum compounds are commonly used for the treatment of a variety of solid tumors: testicular, ovarian,
esophagus, bladder, head and neck and epidermoid cancers in the case of cisplatin and carboplatin, and metastatic colon cancer for oxaliplatin. Their cytotoxic effect is due to the formation of intra-strand (95%) or inter-strand (5%) DNA cross-links (Fig. 12) [48,49], or to the addition of cellular proteins [50,51]. Due to their wide clinical use, resistance to platinum derivatives has been (and is still) the subject of numerous studies which identified multiple mechanisms that could influence tumor response to these agents. These mechanisms have been reviewed recently [52] and will not be described in this paper.

5. The minor groove binding (MGB) alkylating agents

5.1. The peptide-based minor groove binders

With the exception of mitomycin C, classical alkylating agents are primarily targeting the major groove of the DNA. In order to specifically target the minor groove of the DNA, a series of compounds were synthesized based on the chemical structure of two well characterized minor groove binders (MGB), distamycin and netropsin (Fig. 13). These naturally
Fig. 12. (A) Chemical structures of the main platinum derivatives used in the clinic. DACH: diaminocyclohexyl. (B) Intra-strand and inter-strand crosslinks induced by platinum derivatives and their associated frequency. (*) Comp 1 as described in reference [64].

Fig. 13. Chemical structures of the first generation of peptide-based minor groove binders distamycin and netropsin and of derivatives that alkylate DNA and that have been tested in the clinic or show promising anticancer activity.
occurring polypyrrole, crescent-shaped oligoamides were known to interact with sequence-specific region of the minor groove, but had little or no anticancer activity because of their non-covalent binding to DNA. Therefore, various derivatives were synthesized either by adding alkylating groups to the parent MGB, or by substituting pyrrole rings by pyrazole or benzofuran rings, leading to new alkylating MGB with enhanced cytotoxicity [53–55]. The chemical structures of the main derivatives that were tested in clinical trials are shown in Figs. 13 and 14.

Tallimustine (FCE 24517) is a benzoyl nitrogen mustard derivative of distamycin (Fig. 13). Both distamycin and tallimustine were shown to alkylates the N3 of adenines and inhibit the binding of ubiquitous transcription factors such as OTF-1 and NFE1 on specific AT-rich promoter sequences [56,57], or TATA box binding proteins (TBP) leading to a decrease in basal level of transcription [58]. Further mapping of the adduction sites could show that alkylation by tallimustine occurred predominantly on adenines in 5′-TTTTGA sequences in vitro and in cells [59,60]. Interestingly, it was also reported that sequence specificity of distamycin analogs was related to the number of pyrrole rings and associated with the potency of the derivatives, compounds with the higher specificity being the more cytotoxic [61]. These data suggested that selective alkylation of DNA by MGB could be used to inhibit the transcription of specific genes involved in the proliferation of cancer cells.

The development of the haloacryloyl derivative brostallicine (Fig. 13) was also promising because, it had a different mechanism of action as compared to tallimustine, since it was only active in the presence of thiol groups and alkylated DNA in 5′-AAAG sequences [62,63]. Moreover it showed reduced mutagenicity and its activity was retained in MMR-deficient cells and in cells resistant to other anticancer drugs [63]. More recently, new netropsin analogs such as compound 1 (Fig. 13) with reduced mutagenic potential has been identified [64].

CC-1065 and duocarmycin SA (Fig. 14A), are natural antibiotics isolated from *Streptomyces* species that were also identified as specific minor groove alkylators [65]. They also form adenine N3 adducts in AT-rich regions via the cyclopropylpyrrolo[e]indolone (CPI) alkylation unit (Fig. 14A). Their development was abandoned due to bone marrow toxicity and delayed toxicity in mice. Other derivatives such as adozelesin, carzelesin, bizelesin and KW-2189 did not show reduced toxicity either. This led to further characterization of the indolecarboxamide ML-970 (Fig. 14B) that showed reduced mutagenicity [66], or to the synthesis of new heterocyclic carbamate prodrugs such as the seco-CBI-indole2 (Fig. 14B) [67]. The use of seco-CBI-indole2 induces a very slow release of the active free form of the drug, thus allowing

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Fig. 14. (A) Chemical structures of CC-1065 and duocarmycin SA containing the cyclopropylpyrrolo[e]indolone (CPI) unit responsible for DNA alkylation. (B) Chemical structures of their related derivatives. For Bizelesin, R3 corresponds to the moiety shown in the black rectangle.
the administration of much higher doses of the alkylating agent.

Conversely to mice, the severe myelotoxicity of peptide-based minor groove binders that was observed in dogs and to a lesser extent in humans represented the main limitation for drug approval. A direct link between toxicity and N3-alkylation in specific sequences of the minor groove may explain the differences in myelotoxicity patterns between tallimustine and distamycin derivatives [68] or with N2-alkylators such as tetrahydroisoquinolines (see below) that also show a marked hepatotoxicity, the mechanism of which is still unclear. Therefore, continuing efforts to synthesize new compounds with safer therapeutic windows are necessary.

5.2. The illudins

Illudins are natural sesquiterpenes that were isolated from the mushroom Omphalotus olearius (or Clitocybe illudens) in the 1950s (Fig. 15A) [69]. The first illudins to be characterized were named illudins M and S for their antibiotic properties against mycobacteria and staphylococcus strains, respectively (Fig. 15B) [70]. Despite a promising anticancer activity in several tumor models, illudins were not further developed because of toxicity against normal cells. A series of illudin analogs were then synthesized and led to the identification of the acylfulvens (AF) family. Among them, the hemi-synthetic irofulven (hydroxymethylacylfulven, HMAF) was the only derivative to enter clinical trials in 1999, with some responses in pancreatic and prostate cancers but severe dose-limiting toxicities and a lack of efficacy as single agent when the dose was reduced [69,71]. Nevertheless, continuing efforts to synthesize new derivatives are still motivated by (i) a better selectivity of these compounds toward tumor cells as compared to normal cells, (ii) their activity in cells resistant to conventional alkylating agents, (iii) their capacity to potentiate a number of cytotoxic agents in vitro, and (iv) their unique but complex mechanism of action leading to cell death. It is now admitted that AF and HMAF are MGB that preferentially alkylate adenine at position N3, though irofulven can also alkylate N7 of guanines [72]. Conversely to peptide-based MGB, this alklylation is not DNA sequence-specific, and AF and irofulven are also capable to alkylate proteins as well [69,71]. Alklylation by irofulven requires its activation by the NADPH-dependent enzyme 15-oxoprostaglandin 13 reductase (PTGR1) also known as alkenal(one) oxidoreductase (AOR). This leads to the formation of a cyclohexadiene intermediate with a cyclopropyl moiety that is prone to nucleophilic attack by DNA, leading to the formation of mono-adducts that are converted into strand breaks via the formation of abasic sites (Fig. 15C) [69]. It is also interesting to note that cytotoxicity induced by AF or irofulven are closely related to the activity of the transcription-mediated nucleotide excision repair (TC-NER) pathway. This mechanism is similar to what is observed for the tetrahydroisoquinolines (see below) and strongly suggests that besides DNA alklylation, interactions with key factors involved in NER could also play a role in AF-induced cell death [73,74]. Because illudin M and S can be isolated in gram scale from mushroom culture media [69], there is hope that a variety of hemisynthetic derivatives with better tumor selectivity and reduced toxicity could be obtained in the near future [69,71].

5.3. The tetrahydroisoquinolines

This class of alkylating agents emerged in the 1960s following a screening program performed by the NCI to
discover new compounds from marine origin with promising anticancer properties. It was shown at the time that extracts from *Ecteinascidia turbinata*, a tunicate from the Caribbean Sea (Fig. 16A), could inhibit the proliferation of cancer cells in vitro and in xenograft models [75]. However, the active compounds responsible for these anticancer activities were only identified twenty years later and named ecteinascidins. Ecteinascidin 743 (Et743) and its derivatives Et722 and Et729 were the first tetrahydroisoquinolines to be characterized [76]. Several compounds were further developed by Pharmamar. They were first extracted from *Ecteinascidia turbinata* or from the sea slug *Jarunna funebris* (Fig. 16A).

In the case of Et743, extraction from tunicates could not provide enough supply because of low concentrations of active principles, and trabectedin is now obtained by semisynthetic process from the readily available cynosapharin B [77]. The chemical structure of tetrahydroisoquinolines approved in the clinic or in clinical trials is composed of three fused tetrahydroisoquinoline rings (Fig. 16) [78]. Only A and B rings are involved in the interaction with the DNA minor groove. DNA alkylation is due to the presence of the highly reactive carbinolamine center at position 2. The elimination of the hydroxyl group at position 21 leads to an iminium intermediate that is vulnerable to nucleophilic attack by the doublet of guanine N2 [79] (Fig. 16B). Conversely to other N2 alkylators, alkylation by Et743 leads to a DNA bending toward the major groove resulting in extrahelical protrusion of the C-ring that could in turn interact with other cellular proteins [80,81]. This unique alkylation process probably explains the complex mode of action of Et743 that is still not fully understood [78]. Various mechanisms have been proposed to explain the cytotoxic effects of pharmacological concentrations of Et743.

Interference with transcription is certainly playing a crucial role in the cytotoxicity of Et743. It was first demonstrated that the drug could inhibit the binding of specific transcription factors such as SRF/TCF and NF-Y [82], resulting in selective alteration of the transcription of genes with CCAAT-containing promoters such as HSF70 or MDR1 that are quickly downregulated following Et743 treatment [83,84]. This was not observed for distamycin and tallimustine [83]. Et743 also regulates SXR- and Sp1-dependent transcription of MDR1 and other genes involved in drug metabolism such as the cytochrome P450 CYP3A4 gene [85]. Et743 was also shown to regulate the transcription of specific genes involved in cell-cycle though drug treatment had opposite effects depending on the gene. While TK and DHFR transcription was downregulated, expression of E2F and Cyclin E was enhanced by 4–5-fold [86], confirming the complex pattern of expression modulation induced by this drug [87]. It was surmised that protrusion of the C-ring of Et743 into the DNA minor groove could interact with transcription factors and inhibit their binding to promoters. However, absence of the C-ring does not modify the cytotoxicity profile of the tetrahydroisoquinolines as compared to Et743 [88,89]. It is also established that promoter repression is not occurring via the competitive displacement of the transcription factors and that modification of histone acetylation is also not involved in this process [84,86], suggesting that other steps downstream from transcription activation may be altered by a mechanism that remains to be elucidated.

Other mechanisms have been proposed based on DNA strand breaks directly resulting from alkylation [90–92]. These mechanisms rely on the observations that deficiencies in two specific DNA repair systems, Nucleotide Excision Repair (NER) and Homologous Recombination (HR), conferred a drastic alteration in sensitivity to Et743 [90–94]. Deficiency in HR that is involved in the repair of DNA double-strand breaks conferred a higher sensitivity to Et743 [91,94,95], which could easily be explained by the persistence of unrepaired cytotoxic lesions. Paradoxically, deficiency in Nucleotide Excision Repair (NER), that is involved in the repair of UV-induced lesions and in the removal of platinum-DNA adducts, conferred a resistance
Fig. 17. Schematic representation of the signaling pathways that are triggered following DNA alkylation by mono- or bi-functional alkylating agents. The products of mono-alkylation (N- or O-alkylation) with their corresponding base targets are repaired by specific mechanisms: Direct Reversal (DR) involving methyl guanine methyl transferase (MGMT) or human AlkB homologues (ABH) enzymes; BER: Base Excision Repair involving various factors including PARP (poly(ADP) ribose polymerase). PARP inhibition inhibits the repair of adducted bases and sensitizes cells to alkylating agents. Unrepaired DNA adducts can either lead to mutagenic effects because of the persistence of the alkylated base into DNA via the futile cycle of mismatch repair (MMR) (see text for details) or can be transformed into single- or double-strand breaks following replication and lead to cytotoxic effects. DNA intra- or inter-strand cross-links induced by bi-functional alkylating agents are the substrate of Nucleotide Excision Repair (NER) pathway or are eliminated by recombinational repair: homologous recombination (HR) or non-homologous end-joining (NHEJ). If left unrepaired these breaks eventually lead to cell death.

to Et743, suggesting that proficient NER is required for the activity of the drug [91–94]. This is rather counter intuitive since NER-deficient cells are known to be hypersensitive to platinum drugs or UV-induced DNA damage. One hypothesis is that Et743-DNA adducts are recognized by NER factors that are further sequestered onto these lesions and inactivated, leading to repair failure and the formation of lethal DNA breaks [78,89,92]. Indeed, Rad13, the yeast homolog of the human ERCC5 (XPG) NER endonuclease was found in a ternary complex with Et743 and DNA [94]. It was also proposed that the C-ring could be involved in this mechanism, as changes in C-ring structure such as for PM00104 (Zalypsis) could alter the NER-dependent activity of the drug [96].

Several studies also pointed out the role of tumor microenvironment in the response to Et743 as monocytes and tumor-associated macrophages, that play a key role in immunity, were highly susceptible to pharmacological concentrations of the drug [97,98]. In vitro, Et743 inhibited the production of proinflammatory chemokines such as IL-6 or CCL2 as well as other mediators involved in inflammation (cytokines, growth factors) in ovarian and myxoid liposarcoma tumor cell models [97,98]. Using four different mouse models, it was recently shown that Et743 could selectively deplete mononuclear phagocytes in blood and tumor tissues, via the activation of caspase-8-dependent apoptosis, confirming that macrophage targeting is an important component of the antitumor activity of Et743 [99].

Clinically, Et743 (trabectedin, Yondelis®) was approved by EMEA for the treatment of soft tissue sarcomas in 2007. In 2009, it was also approved for the treatment of relapsed platinum-sensitive ovarian cancer. The strategy to use trabectedin in that indication was based on the fact that platinum resistance is often accompanied with an overexpression of NER factors, which could potentiate, at least in vitro, the efficacy of Et743 [78,89,100,101]. Two other derivatives have been developed, PM01183 (lurbinectedin) and PM00104 (Zalypsis®) (Fig. 16A). As compared to Et743, these two derivatives showed similar in vitro cytotoxicity profiles [102].

In vivo, the activity pattern was also similar for PM01183 but was rather different for PM00104 suggesting that its in vivo activity probably involves specific host-mediated mechanisms. PM01183 (lurbinectedin) is in clinical trials for the treatment of lung, breast, pancreatic, ovarian cancers and leukemias and PM00104 for the treatment of multiple myeloma and Ewing sarcomas. Results of these trials are awaited since they will guide the development of this new class of MGB alkylating agents in the near future.

6. Exploiting the signaling pathways induced by DNA alkylation to potentiate drug efficacy

Fig. 17 presents a schematic overview of the signaling pathways that are induced following DNA alkylation. As mentioned earlier, two kinds of DNA adducts need to
be considered. The monoadducts that result from O- and N-alkylation and are repaired by direct reversal (DR). Direct reversal of O-alkylation products (mainly O6-methyl guanines, O6MeG) involves a single enzyme named methyl guanine methyltransferase (MGMT) [103] that catalyzes the transfer of the methyl group onto the thiol residue of its catalytic cystein. Once methylated, MGMT is irreversibly degraded by the proteasome [104]. This mechanism explains that cells maintaining a high level of MGMT expression could resist to alkylating agents such as temozolomide [7,8,11,103]. To overcome this resistance, compounds structurally related to O6MeG such as O6-benzylguanine (O6BG) and its derivatives have been synthesized to serve as a lure for MGMT [103,105]. Association of O6BG with temozolomide results in a significant increase in cytotoxicity due to a decrease in the repair of O6MeG adducts. Though its clinical development did not meet expected hopes, O6BG is the source of research for new derivatives that could be routinely used in combination with classical alkylating agents to potentiate their cytotoxicity [104].

Repair of N-alkylation products on the other hand, involves two mechanisms depending on the nitrogen target. While repair of N1A, N7A and N3C triggers ABH2 or ABH3 enzymes that are homologous to the bacterial demethylase AlkB [106] (Fig. 17), other N-alkylation adducts are repaired by the Base Excision Repair (BER) in which the initial step is the recognition and the excision of the methylated base by N-methyl purine DNA glycosylase (MPG) leading to abasic sites [103,107]. Abasic sites are then cleaved by specific apurinic endonucleases such as APE1. Then, new DNA synthesis allows restoring DNA integrity [108]. There are many studies providing preclinical and clinical evidences that downregulation of BER efficacy by inhibiting key factors such as APE1, polymerase β or the poly (ADP-ribose) polymerase (PARP) could lead to cell sensitization to alkylating agents (reviewed in [109,110]). PARP plays a critical role in the recognition of DNA lesions, especially N7MeG and N3MeA adducts [108,111] and PARP inhibitors have been developed with the idea to potentiate the cytotoxicity of alkylating agents independently of the production of O6MeG [112]. This has been observed in vitro and in xenograft models [113–116] and led to several clinical trials associating temozolomide with various PARP inhibitors (reviewed in [112,117]), some of which providing increased progression-free survival in metastatic melanoma patients [118].

In the case where mono-adducts are not repaired, persistence of alkylated DNA can lead to the accumulation of mutagenic DNA lesions that are ultimately responsible for cell death via the generation of irreversible single- or double-strand breaks (Fig. 17).

Inter- or intra-strand cross-links could also be formed in the case of bi-functional alkylating agents (Fig. 17). These lesions are usually converted into DNA double-strand breaks following replication and are usually processed by one of the two main recombination pathways: homologous recombination (HR) or non-homologous end-joining (NHEJ). In this line, targeting key factors involved in these processes could represent an attractive strategy to enhance tumor response to alkylating agents. Recent studies have provided evidences that downregulation of HR (but not NHEJ) could enhance cell sensitivity to temozolomide in glioma cell lines [119].

Combination therapies have also been envisaged for tetrahydroisoquinolines. As mentioned earlier, the activity of this novel class of alkylating agents is closely related to both NER and HR status of the cells. This explains the particular interest of developing trabectedin in tumors that are refractory to platinum-based therapies (due to the concomitant increase in NER gene expression). Since NER-mediated double-strand breaks induced by trabectedin are repaired by HR, it is reasonable to hypothesize that combination of trabectedin with HR inhibitors could be used to ameliorate drug response. However, this strategy has not been tested in a clinical setting yet.

7. Conclusion

Almost 60 years after the approval of mechlorethamine, one could not imagine that alkylating agents could still be part of the therapeutic armamentarium to battle cancer. Though classical alkylating agents still occupy a major place for the treatment of refractory diseases, new classes of alkylating agents targeting the minor groove of DNA have emerged. The natural origin of these derivatives and their rather unique mechanism of action explain the strong regain of interest in the development of this class of compounds. Of particular interest is the targeting of DNA repair pathways such as transcription coupled NER, a specificity that could be explained by the interaction of DNA adducts with key factors involved in this process. An in depth characterization of the mechanism of DNA and/or protein adduction is probably the next important step to identify derivatives that could target specific DNA sequences and/or specific cellular factors. This could lead to the emergence of new drugs with reduced toxicity and better therapeutic indices that could be used alone or in combination for the treatment of refractory tumors.

Conflict of interest

The authors declare no conflict of interest.

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Biography

Philippe Pourquier, PharmD, PhD, graduated in Pharmacy in 1990 and received his PhD in Biochemistry and Molecular Biology in 1996 from the University of Bordeaux, France. He was appointed as a post-doctoral fellow in the laboratory of Molecular Pharmacology at the National Cancer Institute from 1996 to 2000. He has acquired an extensive expertise in the field of DNA topoisomerases and their inhibitors used in cancer chemotherapy. In 2001 he obtained a senior investigator position at INSERM. He is currently Director of Research at INSERM and is the head of the molecular pharmacology group of the INSERM U916 unit at the Bergonié Cancer Institute of Bordeaux, France, developing basic research programs in the field of drug resistance to topoisomerase inhibitors and alkylating agents. He has recently developed translational research programs to identify alternative therapies for the treatment of castration-resistant prostate cancers and new predictive markers of sensitivity to anticancer drugs that are used or developed in that indication.