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DNA Damaging Agents in Chemical Biology and Cancer

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Abstract: Despite their toxicity, DNA alkylating drugs remain a cornerstone of anticancer therapy. The classical thinking was that rapidly dividing tumour cells left more of its DNA in an exposed single-stranded state, making these rapidly dividing cells more susceptible to alkylating drugs. As our understanding of DNA repair pathways has matured it is becoming clear that compromised DNA repair – a hallmark of cancer – plays a role as well in defining the therapeutic window of these toxic drugs. Hence, although new alkylating motifs are unlikely to progress through the clinic, the legacy of these medicines is that we now understand the therapeutic potential of targeting DNA damage repair pathways. Here we look at the history of alkylating agents as anticancer drugs, while also summarizing the different mechanistic approaches to covalent DNA modification. We also provide several case studies on how insights into compromised DNA repair pathways are paving the way for potent and less toxic targeted medicines against the DNA damage response.

Keywords: DNA alkylation · Cancer · Chemotherapy · Covalent drugs · Nucleic acids

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Basilius Sauter was born in 1989 in Basel and started his chemistry studies with an apprenticeship as a lab technician in medicinal chemistry. Later, he studied molecular life sciences at the FHNW before he switched for his master's degree to the University of Basel. There, he joined the group of Prof. Dennis Gillingham in 2016 to pursue a PhD in chemical biology, originally studying the effect of DNA alkylating drugs on RNA. He completed his degree in 2020 and is now continuing his projects in the same group. He is currently involved in projects around DNA encoded libraries and applications of Next Generation Sequencing in chemical biology.

1. An Overview of Alkylating Anticancer Drugs

"*Alle Dinge sind Gift, und nichts ist ohne Gift; allein die Dosis machts, dass ein Ding kein Gift sei.*" Paracelsus, 1538.[1]

Nothing reflects this quote from Paracelsus as well as the alkylating anticancer drugs do. Mustard gas was used in the First World War as a chemical weapon to kill enemy soldiers, and yet these molecules had effects that led Dr. James Ewing to hypothesize that they might be helpful in treating neoplastic growth. Based on his suggestion, Frank Adair and Halsey Bagg reported in 1931 in the *Annals of Surgery* a first success in treating skin cancer by repeated application of a 20% mustard gas solution in ethanol.[2] The high toxicity of this substance, however, permitted only dermal applications. A decade later, treatment of different cancers by intravenous injections of a nitrogen mustard (**1a**) was shown, achieving effects comparable to radiation therapy.[3] Everett and Ross systematically tested different arylnitrogen mustards and found that two chloroethyl groups are essential for activity, and that activity correlates with the rate of hydrolysis.[4] These publications marked the beginning of the development of anticancer drugs with lower toxicities to the patient.[4,5] Of course, compared to today's targeted medicines these molecules are still incredible poisons, yet in many cases they remain some of the most effective treatments. For example, the alkylating agent temozolomide (**6a**) is the only approved first-line drug against glioblastoma.[6]

1.1 *Mechanisms of DNA Alkylating Agents*

Nowadays, there are several different classes of alkylating antineoplastic drugs unrelated to mustard gas. The World Health Organisation (WHO) distinguishes five specific classes in their Anatomical Therapeutic Chemical (ATC) Classification System, each having their own distinct mechanisms.[7] The oldest family are the aforementioned nitrogen mustards analogues **1**, of which all have the bis(2-chloroethyl)amine moiety in common (Fig. 1). Other major categories include the alkyl sulfonates **2**, aziridines **3**, nitrosoureas **4**, and epoxides **5**. Drugs that are known to be alkylating agents like temozolomide (**6a**), but can't be grouped together with the others are listed as 'others'. Although platinum reagents such as cisplatin (**7**) do modify DNA, they do this through metal chelation, which distinguishes them from alkylating agents.

The oldest member of the nitrogen mustard drug family **1** is chlormethine (**1a** in Fig. 1), the only chemical weapon to find clinical use.[8] Chlormethine and related molecules form a reactive aziridinium, which is the active alkylating agent. The mustard **1a**, however, is not a good prodrug because solutions must be made fresh due to its high hydrolysis rate. Molecules with aniline-type nitrogens like chlorambucil (**1b**) and bendamustine (**1c**) were developed to solve this problem and can be given orally.[9] The electron-withdrawing property of the aryl ring reduces the ability to

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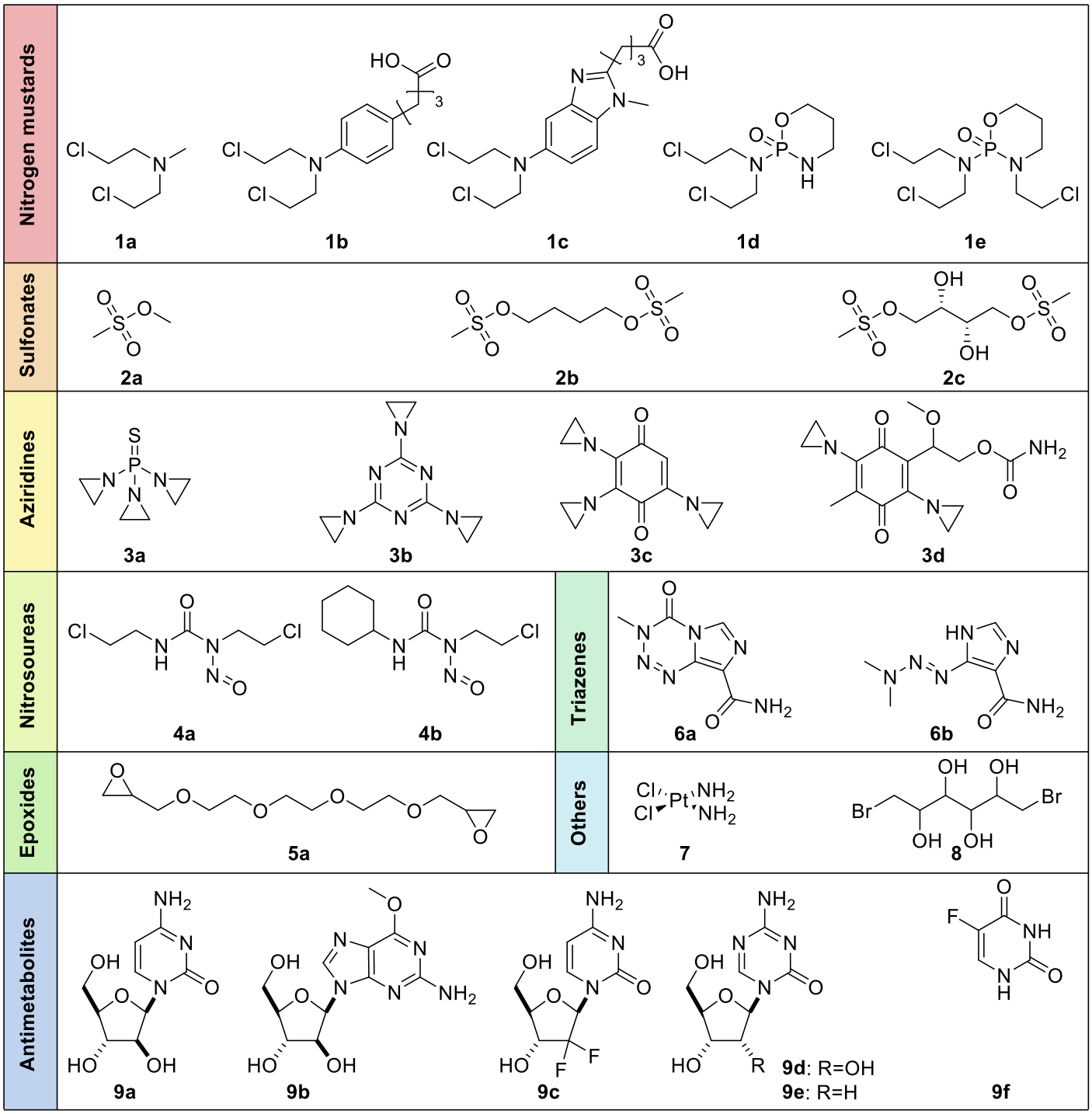

Fig. 1. Chemical structures of some of the DNA alkylating drugs and cisplatin.

form the reactive aziridinium (Fig 2, top left). To further prevent early hydrolysis, cyclic phosphamides such as cyclophosphamide (**1d**) or trofosfamide (**1e**) have been developed that first need to get activated *in vivo* before they can react with DNA.[10]

Aziridines (**3**) were designed to mimic the reactive species of nitrogen mustards by skipping the intermolecular S_N2 reaction. They are mainly protonated at pH 7.4 and thus very reactive. For this reason, available therapeutics such as thiotepa (**3a**) or triethylenemelamine (**3b**) make use of electron-withdrawing groups to reduce the basicity of the aziridine and consequently their reactivity and toxicity. Some drugs such as triaziquone (**3c**) or carboquone (**3d**) actively use this effect to allow for *in vivo* activation. Their benzoquinone motif gets reduced in cells and loses its electron-withdrawing properties, thus increasing the reactivity.

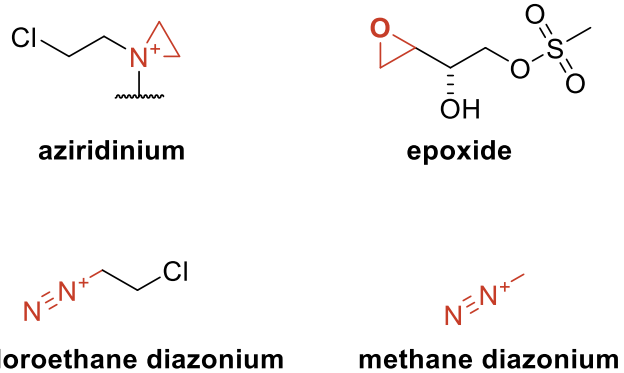

Fig. 2. Activated electrophiles of DNA alkylating drugs.

Despite their chloroethyl group, nitrosoureas **4** are conceptually different. Instead of forming an aziridinium as their intermediate step, they decompose into isocyanates (which can carbamoylate proteins),[11] and a diazonium compound (Fig 2, bottom left) – a potent electrophile that will attack nearly anything, but is particularly toxic when it attacks DNA.[12] If the nucleophile is a nitrogen, the resulting product likely forms an aziridinium, enabling it to form crosslinks. Some clinically relevant members of this family are lomustine (**4a**) and carmustine (**4b**). Triazenes **6** such as temozolomide (**6a**) and dacarbazine (**6b**) use a similar mechanism, but are unique as they transfer only a methyl group and cannot form crosslinks.

Other DNA alkylating electrophiles that have formed the basis of drugs include sulfonates and epoxides. For sulfonates we have methyl methanesulfonate (**2a**),[13] busulfan (**2b**, its two sulfonate groups allow it to form crosslinks, like the nitrogen mustards),[14] and treosulfan (**2c**, an active intermediate formed by intramolecular substitution is shown in Fig 2, top right). Representative members of the epoxides include mitobronitol (**8**, *via* the intramolecular formation of an epoxide) and etoglucid (**5a**), although there are many natural products, such as azinomycin and aflatoxin, that target DNA through a reactive epoxide as well.[15]

1.2 *DNA and RNA Damage with Nucleotide Antimetabolites*

Instead of adding electrophiles to damage DNA after replication in order to cause havoc, another approach is to use nucleotide-like molecules that infiltrate the DNA synthesis pathway, so-called antimetabolites. In cancer treatment, antimetabolites have found wide usage and we highlight several representative cases here.[16] Cytarabine (**9a**) and nelarabine (**9b**) are arabinose analogues to cytosine and guanine respectively. The inversion of the 2'-hydroxyl is enough to cause polymerase stalling,[17] which, if left unrepaired, can lead to initiation of apoptosis. Gemcitabine (**9c**) is another cytosine analogue with two fluorines on the 2' position. After conversion to the corresponding triphosphate, it gets incorporated during replication as well. In contrast to cytarabine (**9a**), it allows the incorporation of one other nucleotide before it stalls replication,[17b] allowing it to avoid normal DNA repair.[18] Azacytidine (**9d**) and decitabine (**9e**) differ from their natural nucleoside counterparts through a nitrogen substitution at position five. This change can inhibit protein synthesis upon incorporation into RNA (mainly for azacytidine (**9d**), which inhibits transfer RNA cytosine-5-methyltransferase),[19] or can cause hypomethylation of CpG islands (mainly for decitibine (**9e**), which inhibits DNA methyltransferases).[20] Fluorouracil (**9f**) on the other hand inhibits thymidylate synthase, preventing the methylation of dUMP to form dTMP,[21] which ultimately leads to depletion of dTTP and finally cell death. Faulty integration of fluorouracil into RNA may also be a contributing mechanism to its toxicity.[22]

As the above examples demonstrate, whatever the mechanism, DNA-damaging agents form the basis of some of the most potent anticancer agents. Molecules like these have also enabled cell biologists to study why it is that seemingly potent and indiscriminate toxins can have a functional therapeutic window. General replication stress and compromised DNA damage repair (collectively termed the DNA damage response (DDR)) are now considered hallmarks of cancer. The compromised DDR in transformed cells makes them more vulnerable to DNA damage. But of course, such potent poisons are still generally toxic, leading to many of the horrific side effects we associate with chemotherapy. Can we decouple the therapeutic efficacy from toxicity? As we will discuss at the end, there seems to be great potential here. Taking inspiration from the DNA damaging medicines, researchers today are continuing to focus on the DDR, but targeting critical proteins in the pathway rather than using the blunt instrument of DNA damage. Before we look at this however, we present how the chemistry of DNA and RNA damage has underpinned some important advancements in biotechnology and chemical biology.

2. DNA and RNA Modifications in Chemical Biology

The nucleobases of DNA and RNA are full of nucleophilic centres, varying in strength depending on the atom and its surroundings. Furthermore, the phosphate backbone is also able to act as a nucleophile, as does the 2' hydroxy group of RNA. Beranek has an excellent review of the reactivities of different types of methylating and ethylating agents.[23] Additionally, we have provided a comprehensive discussion of how to understand the reactivity of DNA and RNA bases with different electrophiles.[24] In short, alkylation rates correlate with the nucleophilicity of the base, but also depend on the alkylation agent, the solvent accessibility, and neighbouring bases. Of course, natural evolution would disfavour the adoption of highly reactive structures in DNA and RNA, since this would introduce the possibility to corrupt the information stored and transferred with these molecules. Nevertheless, using simple reactive electrophiles, or more complex chemical warheads (such as those outlined in the previous section) chemical biologists have devised ways to use chemical reactivity to study DNA and RNA biology.

Chemical derivatization enabled early DNA sequencing. Maxam and Gilbert published in 1977 a chemical sequencing method that was superior to the older biochemical plus/minus method from Sanger and Coulson.[25] They separated a double stranded, 5' ^{32}P-labelled 64 bp long piece of DNA with a known sequence and separated both strands on a polyacrylamide gel. Then, they treated both strands individually with their set of reactions that led to selective breakage at specific bases. As only one end of the strand is radioactively labelled, separating on a polyacrylamide gel allowed them to read out the sequence. One of the key reactions was the treatment with dimethylsulfate (**10**, DMS). This led to a mixture of 7mG and 3mA which both result in a positive charge that weakens the glycosidic bond. Heating at neutral pH easily results in an abasic site that, upon treatment with strong base at high temperature, leads to the breakage of the strand. As the 7N of guanine is more nucleophilic, this reaction leads to preferential cleavage at G sites, which differentiates G from A. By using milder conditions, it is possible to cleave the weaker glycosidic bond of methylated A selectively which leads to more strand breakages at A, complementing the reaction set and making it possible to distinguish As and Gs.

2.1 *RNA Probing*

In 1980, Peattie and Gilbert sequenced terminally ^{32}P-labelled tRNA at different stages of denaturing with methods related to Maxam-Gilbert sequencing.[26] The treatments with DMS (**10**) and diethylpyrocarbonate (**11**, DEPC) preferably modify bases not involved in Watson-Crick base pairing or tertiary interactions, which in turn lead to strand breakages. These breakages can be sequenced by gel electrophoresis to determine the position and corresponding base. The strand breaks did indeed correlate with the most solvent accessible positions in known crystal structures of yeast tRNA^{Phe}.[27]

A method described by Stern *et al.* expands upon the idea to use sequencing to determine secondary and tertiary interactions,[28] but utilize the effect that reverse transcriptases have difficulties overcoming kinetic barriers introduced by nucleobase alkylations, an effect originally discovered by Youvan and Hearst.[29] They utilized DMS to form methylations at N7-G, N1-A and N3-C, of which only N7-G does not inhibit primer extension except when the RNA is additionally treated to cause specific strand breaks. Kethoxal (**12**, KE) was added to react at N1-G and N2-G, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (**13**, CMCT) to cause additional inhibiting modi-

fications at N3-C. Both KE (**12**) and CMCT (**13**) react just like DMS (**10**) only with nucleobases not involved in hydrogen bonding.

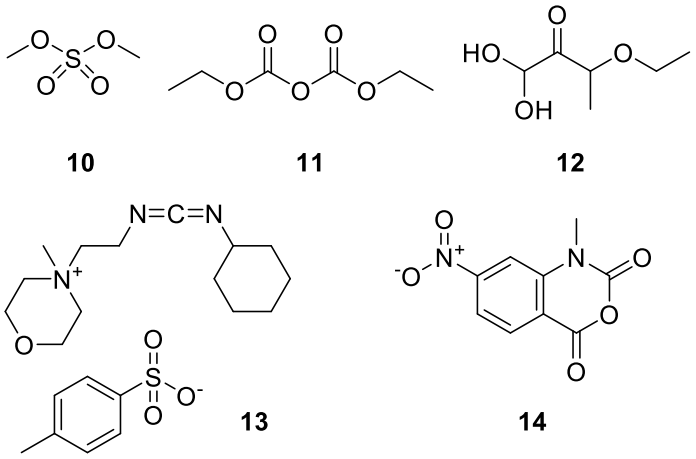

Fig. 3. Electrophiles used in chemical biology to determine secondary and tertiary structures of RNA.

2.2 *Transcriptome-wide RNA Probing*

With the advent of Next Generation Sequencing (NGS), several research groups adapted these protocols to the new sequencing platforms, increasing throughput and allowing the parallel interrogation of the whole transcriptome.[30] All these techniques begin with a modification reaction that targets the most sensitive regions of a folded RNA (typically single-stranded or structural transition regions). Once the RNA is modified or fragmented, cDNA synthesis then creates copies where the ends report on the lesion sites in the original RNA. In most methods DMS (**10**) is used for RNA modification, which causes polymerase stalling at methylated As and Cs, but not at Gs and Us. An alternate technique to probe tertiary structures of RNA that does not rely reverse transcriptase stalling is hydroxyl radical foot printing (HRF). Here, the solvent accessible backbone of RNA is reacting with *in situ* produced hydroxyl radicals, either from an X-Ray source or from the reaction of Fe(II)-EDTA with hydrogen peroxide. The radical removes a hydrogen from either the C4' or C5' of the ribose, leading to the cleavage of the RNA strand.[31] Perhaps the most exciting of these new whole-transcriptome RNA probing approaches is a variant of SHAPE-Seq (selective 2'-hydroxyl acylation analysed by primer extension),[32] which uses a reagent (1-methyl-7-nitroisatoic anhydride (**14**, 1M7)) that selectively modifies solvent exposed 2' hydroxy groups. These modifications also block reverse transcriptase progression, leading to truncated cDNA.[33] Newer methods overcome some of the problems associated with reverse transcription stops by relying on mutational profiling instead.[34]

2.3 *Nucleoside Antimetabolites for Studying DNA and RNA Turnover*

A whole different approach is the introduction of DNA and RNA antimetabolites to label cellular DNA and RNA. 5-Bromouridine has been introduced in combination with immunoprecipitation and NGS to measure the half-live of individual RNA, revealing that ncRNA and housekeeping mRNA have a much longer half-life compared to other types of RNA.[35] 5-Ethynyl-2'-deoxyuridine (EdU) has been used to label DNA with the help of fluorescent azides *via* the Cu(I)-catalysed alkyne azide cycloaddition. Its incorporation can be used to highlight freshly synthesised cells to study cell proliferation and has been shown to work in mice.[36] EdU has also been used in a technique coined iPOND (isolation of proteins on nascent DNA).[37] Here, cells are treated with EdU and shortly after, formaldehyde is added to crosslink proteins to DNA. By clicking biotin on DNA incorporating the antimetabolite, the purification of proteins at replication forks is possible. Consequently, by changing the EdU pulse time the history of which proteins are involved at which time point during DNA replication can be retrieved. The RNA analogue of EdU has been used for observing RNA turnover, too, showing that transcription rates vary greatly among tissues and cell types.[38] More recent, Luedtke and co-workers developed less-toxic arabinose-based alternatives that only show little to no cellular arrest, enabling long-term studies with greater sensitivities.[39] They injected (2'S)-2'-deoxy-2'-fluoro-5-ethynyluridine in Zebrafish embryos in order to birth-date DNA.

3. New Approaches to Target the DNA Damage Response in Cancer

Although it is now widely accepted that compromised DNA repair and its associated replication stress is a hallmark of cancer,[40] the clinical success of DNA alkylating agents and antimetabolites preceded this understanding. We now appreciate that genetic instability is required for cancers to collect the somatic mutations that promote the transition to neoplasticity. But genetic instability comes at a cost: cancer cells cannot repair DNA as effectively as untransformed cells. This feature is likely responsible for the therapeutic window of DNA antimetabolites, alkylating agents, and topoisomerase inhibitors. Armed with this understanding researchers are now studying whether we can achieve targeted inhibition of critical proteins involved in the DNA damage response. In this way it might be possible to recapitulate the apoptotic effects of DNA alkylating agents, without the toxicity associated with injecting or ingesting large amounts of potent poisons.

3.1 *Case Studies of Approved Medicines that Exploit Deficiencies in DDR*

The most instructive example of exploiting cancer vulnerabilities that arise from genetic instability is the development of PARP inhibitors against BRCA1/2 mutated cancers (leaving cells unable to carry out homologous recombination repair).[41] In normal cells PARP inhibition is not particularly toxic. Since PARP is responsible for early detection and signalling of DNA single-strand breaks, the resilience of normal cells is likely attributable to various back-up DNA repair pathways that can shoulder the burden of inactive PARP. If other DNA repair pathways are compromised, however, PARP inhibition becomes especially toxic. These types of conditional toxicity are called synthetic lethalities and they have been appreciated for a long time.[42] It is unsettling to think that PARP inhibitors were nearly abandoned[43] because early clinical trials showed poor efficacy. Only once responses across different genetic subtypes were considered, was the potency of PARP inhibitors unveiled.

Another case of a vulnerability created by mutations in DNA repair is seen in patients undergoing cisplatin treatment for non-small cell lung carcinoma. Patients with upregulated NER (as measured by the expression levels of an excision repair associated protein XRCC1) have better overall survival if their tumours have low expression of BRCA1.[44]

Still a third example comes from glioblastoma, an aggressive malignancy with a dismal 5-year survival rate.[45] The alkylating agent temozolomide coupled with radiation treatment leads to an increase in overall survival of several months. The DNA damage induced by temozolomide is quickly repaired by the direct DNA repair protein methylguanine methyltransferase (MGMT). Promoter methylation can lead to silencing of MGMT (yet another way that cancers achieve genetic instability) and this turns out to occur in nearly 50% of newly diagnosed glioblastomas. Patients whose tumours suffer this type of genetic instability respond much better to treatment with temozolomide because they cannot effectively repair the alkylation damage.[6,45c]

The examples above point the way to a future where a detailed knowledge of cancer genetics could help clinicians personalize treatment regimens. For drug developers these cases underscore the importance of functional screens that search for cancer-specific vulnerabilities. In this sense the emergence of the CRISPR/Cas9 technology is timely. Genome-wide knock-out screens in cancer cell lines (which, as already discussed, typically have a compromised DNA damage response system) has allowed the identification or validation of synthetic lethal combinations with unprecedented speed.[46] Hybrid functional genomics/small molecule screens are a recent innovation,[47] which might identify novel vulnerabilities for which we already have small molecules.[48] In our opinion, combining functional genomics with classical small molecule screening is an area poised for rapid development.

3.2 *New Approaches to Targeting the DDR*

Cycling cells have many checkpoints to insure faithful and complete cell division once the cell has committed. Cells that have faulty DNA repair and/or are experiencing large amounts of damage (such as with chemotherapy) frequently fail checkpoint controls and enter into a fragile state where replication is halted until the problem is fixed, or apoptosis is initiated.[49] This situation would recommend two approaches for exploiting the DNA damage response (or more generally any type of replication stress):[40c,50] targeting proteins that are normally not essential, but become so when checkpoints are activated (a frequent occurrence in cancer cells). A second approach is to combine classical DNA damaging agents or radiation therapy (which both initiate damage checkpoints) with inhibitors of critical checkpoint proteins such as ATM[51] and ATR.[52]

Our understanding of the therapeutic window of DNA damaging agents has evolved considerably in recent years as cancer geneticists and cell biologists have outlined a clearer picture of the DDR. Chemists have historically put much emphasis on the development of warheads or antimetabolites that create DNA damage. Through a more sophisticated understanding of the DDR, today we appreciate that the same therapeutic benefits might be possible by targeting instead critical proteins in the DDR.[53] Moreover, by combining damaging agents with DDR inhibitors severe toxicities can be created in cancers (which already have compromised DDR components (*e.g.* mutations, deletions)), while leaving normal cells unscathed. These insights have galvanized medicinal chemists to understand the DDR and its every druggable component.

Throughout the nearly one hundred years since chemical poisons were first rubbed on melanomas, DNA damaging agents have been a cornerstone of chemotherapy. Medicines that selectively kill cancers by exploiting their genomic instability (*i.e.* compromised DDR) are an exciting prospect that might make direct DNA damaging agents obsolete. Realistically, smart new combination therapies will be the next advance. These will be combinations not borne out of empirical "try it and see" approaches, but rather from a clear biological hypothesis about treatment synergies.[54]

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