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Perspective

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DNA Adducts from Anticancer Drugs as Candidate Predictive Markers for Precision Medicine

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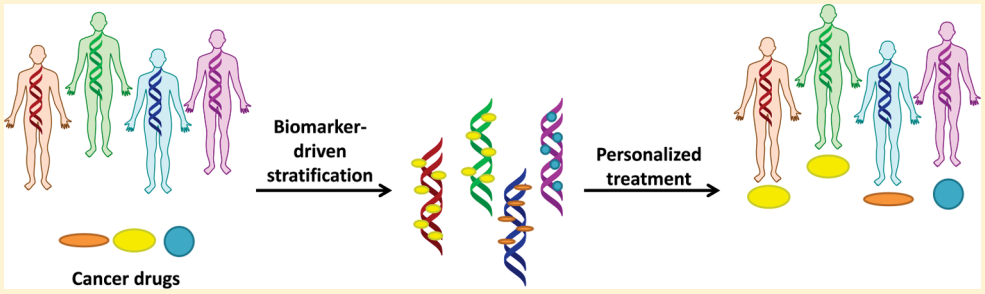

ABSTRACT: Biomarker-driven drug selection plays a central role in cancer drug discovery and development, and in diagnostic strategies to improve the use of traditional chemotherapeutic drugs. DNA-modifying anticancer drugs are still used as first line medication, but drawbacks such as resistance and side effects remain an issue. Monitoring the formation and level of DNA modifications induced by anticancer drugs is a potential strategy for stratifying patients and predicting drug efficacy. In this perspective, preclinical and clinical data concerning the relationship between drug-induced DNA adducts and biological response for platinum drugs and combination therapies, nitrogen mustards and half-mustards, hypoxia-activated drugs, reductase-activated drugs, and minor groove binding agents are presented and discussed. Aspects including measurement strategies, identification of adducts, and biological factors that influence the predictive relationship between DNA modification and biological response are addressed. A positive correlation between DNA adduct levels and response was observed for the majority of the studies, demonstrating the high potential of using DNA adducts from anticancer drugs as mechanism-based biomarkers of susceptibility, especially as bioanalysis approaches with higher sensitivity and throughput emerge.

CONTENTS

1. INTRODUCTION

1.1. Precision Medicine in Cancer Chemotherapy. Selecting a cancer treatment according to physiological characteristics of the disease, such as the organ where the cancer is present, is an approach that views cancer as a homogeneous disease and has become outdated as a result of deeper understanding gained in the past decades regarding the biology and molecular heterogeneity of cancers.[1−3] This

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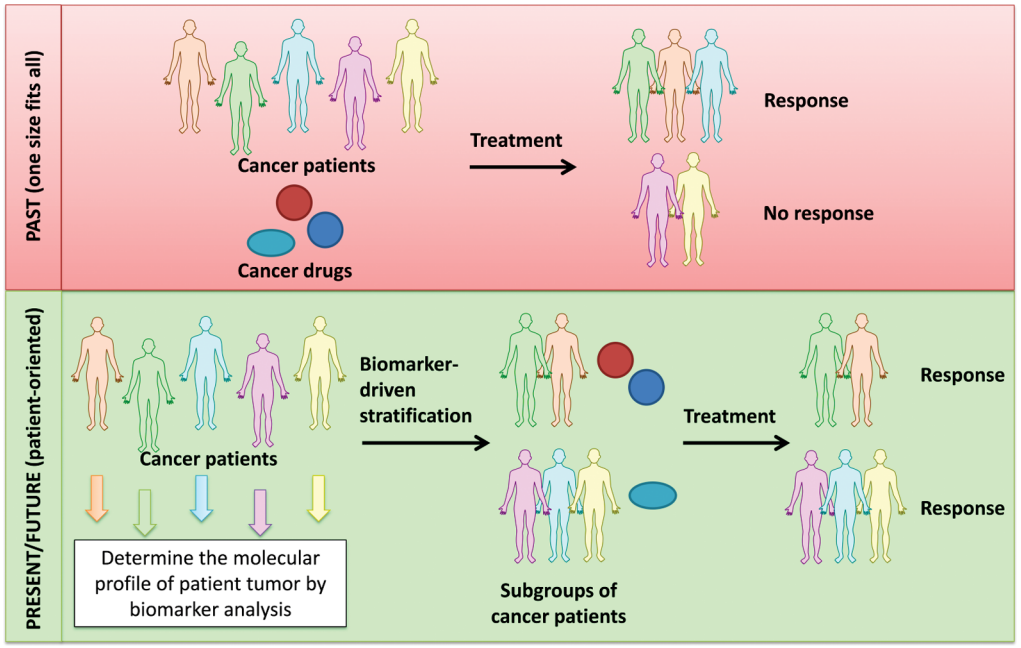

Figure 1. Shifting the focus of cancer therapy by implementation of biomarkers in precision medicine.

Table 1. Biomarkers Used in Modern Cancer Therapy[a]

biomarker category	definition	example(s)
susceptibility/risk	indicates the potential for developing a disease or medical condition or sensitivity to exposure in an individual without clinical apparent disease or medical condition	BRACA1/2 mutations for breast cancer, infection with certain HPV subtypes for cervical cancer
diagnostic	identifies individuals with the disease or condition of interest or to define a subset of the disease	blood sugar or HbA1c to identify diabetes mellitus, serum creatinine, or GFR to identify patients with kidney failure
monitoring	used to detect a change in the degree or extent of disease; may be also used to indicate toxicity or assess safety, or to provide evidence of exposure (including medical products)	PSA when assessing patients with prostate cancer to evaluate disease status of burden
prognostic	used to identify likelihood of a clinical event, disease recurrence, or progression	BRCA1/2 to evaluate likelihood of a second breast cancer, PSA to assess likelihood of cancer progression
predictive	used to identify individuals who are more likely than similar patients without the biomarker to experience a favorable or unfavorable effect from a specific intervention or exposure	DNA adducts
pharmacodynamic/response	used to show that a biological response has occurred in an individual who has received an intervention or exposure	INR when evaluating a patient's response to warfarin treatment
safety	used to indicate the presence or extent of toxicity related to an intervention or exposure	hepatic aminotransferases when evaluating hepatotoxicity

[a]Abbreviations: HPV, human papilloma virus; BRCA1/2, breast cancer genes 1 and 2; GFR, glomerular filtration rate; HbA1c, hemoglobin A1c; INR, international normalized ratio; PSA, prostate specific antigen.

paradigm shift means that patients with the same type of cancer may have different genetic alterations in their tumors that may explain why patients with phenotypically similar cancers may respond to or resist differently the same anticancer treatment.[3] Thus, cancer therapy is undergoing a major shift from one size fits all to precision, patient-oriented personalized approaches. In the future, genetic defects and metabolic features in an individual cancer will drive treatment selection (Figure 1).[3]

Precision chemotherapy is supported by the discovery, development, and validation of biomarkers. These measurable chemical or molecular indicators of biological processes or pharmacological responses can inform therapeutic strategy.[3,4] Clinicians use biomarkers to identify target patient populations, to predict drug efficacy and patient response, resistance, and toxicity, and to stratify patients on the basis of their likelihood to respond to a particular therapy.[5] In general, biomarkers can be divided into seven categories: susceptibility/risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamics/response, and safety (Table 1). Of particular interest in the context of precision cancer chemotherapy are predictive biomarkers, which are used to identify individuals who are more likely than similar patients lacking the biomarker to experience a favorable or unfavorable effect from a specific intervention or exposure.

Knowledge of biomarker levels can inform clinicians regarding which drugs to administer. In cancer therapy, typically a two- or three-drug cocktail is used that covers different molecular targets, reducing the risk of clonal selection based upon cell resistance to a single drug. Drugs that directly modify DNA bases or form cross-links in DNA such as platinum-based or nitrogen mustard drugs are often used as a component of cancer drug combinations, with proven effectiveness in eliminating cancer during their more than 60 years of use.[6−9] The main mechanism of toxicity for the

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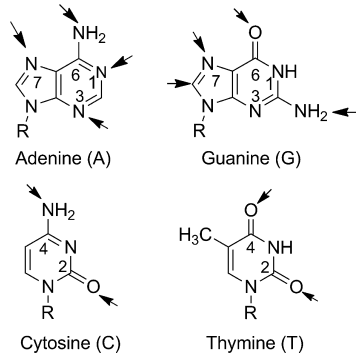
anticancer activity of DNA damaging agents involves the induction of DNA modifications[7,8] that interfere with replication, an overactive process in rapidly dividing cancer cells. Drawbacks of the DNA damage strategy is the possibility for developing resistance, and the high rate of side effects caused by damaging DNA or possibly other critical biomolecules in nontarget cells.[7] In the paradigm of precision medicine, the advent of DNA damaging drug-specific biomarkers is anticipated as a tool for stratifying patients and identifying factors directly relevant to cellular DNA damage that may be exploited for improving chemotherapy with DNA-modifying drugs.

There are three exposure scenarios that can be envisioned for monitoring DNA adducts as predictive markers for DNA damaging cancer drugs. The first involves patients undergoing a first round of chemotherapy, and biological samples such as tumor biopsy, circulating tumor cells (CTCs), and surrogate tissues, are collected and analyzed to reflect actual levels of adducts formed *in vivo* at therapeutic doses. A second scenario involves treating patients with a microdose of the DNA alkylating drug and collecting peripheral blood mononuclear cells (PBMCs) or tumor biopsy for adduct analysis. Finally, a third scenario involves *ex-vivo* exposure of cancerous or normal surrogate cells to the drug, and evaluating adduct formation. Difficulties associated with using DNA adducts as predictive markers centers on the fact that they are present at extremely low-abundance making isolation from complex matrices such as blood, tissues, and urine, and their subsequent detection very challenging.

Research addressing the relationship between DNA adduct levels and the sensitivity of cells grown in culture, animal models, or cancer patients has been explored over the past 30 years (summarized in Tables 3 and 4). In this perspective, we focus on progress regarding establishing an understanding of the relationship between DNA damage and cellular responses to drugs, and the feasibility of using DNA adducts as predictive biomarkers in cancer chemotherapy. We consider preclinical and clinical data concerning DNA damaging drugs that have been used in cancer chemotherapy for decades, such as platinum drugs and nitrogen mustards, as well as experimental drugs that have undergone or may soon enter clinical evaluation, such as acylfulvenes and hypoxia-activated prodrugs. An inclusion criterion for the examples presented herein was the availability of data aiming to define a relationship between adduct formation and cellular responses to the drug. Challenges and opportunities of future development of using DNA adducts as predictive biomarkers in precision medicine are discussed.

1.2. Formation and Relevance of DNA Adducts from Anticancer Drugs. Endogenous and exogenous electrophilic reactive molecules can chemically react with cellular DNA, such as by alkylation, arylation, arylamination, oxidation, deamination, and cross-linking reactions, and nucleophilic sites in DNA are commonly modified by the addition to electrophilic molecules.[8,10,11] The reactive sites in DNA include especially the *N*7, *N*2, and *O*6 positions of guanine; *N*1, *N*3, and *N*7 positions of adenine; the *O*2 and *O*4 positions of thymine; and the *O*2 and *N*4 positions of cytosine (Scheme 1).[12] Adducts may also arise from modification at the *C*8-position of purines, either via radical-mediated reactions or migration after reactions at nucleophilic sites. Because of the high number of possible modification positions and different electrophiles, there is a large diversity of DNA adduct structures; therefore, a first question to address in the study of DNA modifying drugs concerns the characterization of DNA adducts that may be responsible for inducing cell death.

Scheme 1. Nucleobase Sites in DNA That Are Frequently Modified by Electrophiles. R = 2′-deoxyribose



There are several determinants of sensitivity to drug-induced DNA adducts in cancer cells. First, the efficiency with which DNA adducts are formed depends on relative rates of transport and metabolism processes that compete in the uptake and activation vs detoxification and efflux of anticancer drugs. Additionally, the potency of a given adduct can relate to its chemical structure, for example, monoadducts are generally anticipated to be less toxic than cross-linked adducts. Furthermore, biological stability vs decomposition and repair susceptibility influence the capacity for adducts to persist and inhibit replication and transcription, induce double strand breaks, and initiate apoptosis. High DNA damage levels are sensed by regulatory cell cycle proteins whose activation induces either arrest of the cell-cycle for DNA repair or apoptosis to prevent transmission of the DNA damage during mitosis. DNA lesions occurring in the S-phase of the cell cycle can block replication fork progression leading to replication-associated DNA double strand breaks (DSBs), one of the most deleterious lesions that can occur in DNA. Greater persistence of the DNA damage can therefore more efficiently result in cell death. Greater efficiency of treatment is reached, therefore, when DNA adducts are higher or confined to cancer cells, and poorly repaired. Conversely, resistance and treatment failure may arise if DNA damage is repaired, and side effects arise from high damage in noncancerous cells (Scheme 2).[13,14]

1.3. Measuring DNA Adducts. As chemical biomarkers, DNA adduct levels have the unique advantage of being an integrative measure of a plethora of potential drug resistance mechanisms including pharmacokinetics, tumor microenvironment, drug uptake/efflux, metabolic drug inactivation, adduct formation, and DNA repair. However, technical challenges include the fact that DNA adducts are usually present in very low abundance compared to unmodified nucleobases, often in the range of 0.01−10 adducts per 10^8 unmodified nucleobases.[15−18] For this reason, highly sensitive, accurate, and specific approaches, often involving specialized instrumentation such as mass spectrometers, are used for detection and quantitation of low abundant adducts in DNA extracted from tissues, cells, blood, and saliva, or nucleobase adducts excreted in urine.[19−21]

Many early studies of DNA alkylation relied on alkaline elution or the alkaline comet assay. The alkaline elution technique, developed by Kohn and co-workers in the early 70s, is one of the earliest methods for detecting DNA damage. With it, DNA single strand breaks are measured on the basis of the

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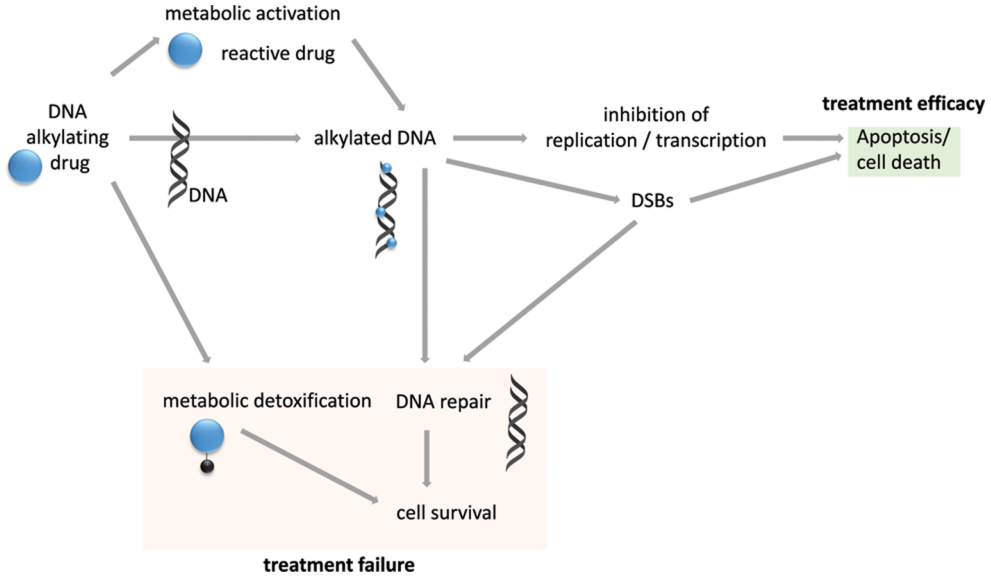
Scheme 2. Biological Relevance of DNA Adducts Induced by DNA Alkylating Drugs



rate DNA fragments of differing size elute through a filter membrane under alkaline conditions.[22−24] The alkaline comet assay is used to detect double and single-strand breaks, and under certain conditions, it can be used to detect also DNA−DNA and DNA−protein cross-linking, on the basis of DNA migration in an agarose matrix under electrophoretic conditions.[25] Both of these assays are relatively easy to implement. However, they have the drawback of measuring overall DNA single strand breaks or DNA cross-linking rather than chemically specific DNA adducts, which makes it more difficult to relate cellular or clinical response to the DNA alkylating drug. Accordingly, these two techniques are rarely used in a clinical setting.[26,27]

Other techniques include atomic absorption spectroscopy (AAS), radiolabeling, ^{32}P-postlabeling, and immunoassays. AAS has been extensively used for determining platinum-DNA adducts. The principle of AAS relies on the ability of metal atoms to absorb strongly at characteristic wavelengths, which coincide with the emission spectra lines of the particular metal.[28] Classically, radiolabeling, ^{32}P-postlabeling, and immunoassay techniques were used and are explained in more detail below.

In modern research, the methods mentioned above have been largely surpassed by mass spectrometry approaches (Table 2). Depending on the abundance of the lesion and on the sensitivity of the technique used, between 1 and 200 μg of DNA are usually required for DNA adduct analysis by mass spectrometry.[20,21,29] Choice of method depends on the type of sample, the nature of the investigation, as well as practical considerations balancing content of information with resources available.[29]

1.3.1. Radiolabeling and ^{32}P-Postlabeling. Radiolabeling is a very sensitive technique that has been used to detect as little as 1 adduct in 10^9 nucleotides.[30] DNA adduct determination with radiolabeled compounds is usually conducted by administering a single dose of the radiolabeled test substance to laboratory animals. After completion of the experiment, which can last hours to days, animals are sacrificed, DNA is isolated from organs or cells, and the amount of radioactivity in the test vs control DNA sample is determined by liquid scintillation counting (LSC).[29−31] For this approach, it is essential that the isotope labeled atoms are in positions resistant to loss during drug metabolism or adduct formation. The use of radiolabeling for measuring DNA adducts is often limited by the high cost and effort for the synthesis of labeled compounds, as well as related to safe handling and disposal of materials.[21,29]

^{32}P-postlabeling is a highly sensitive technique first introduced for DNA adduct detection in the early 1980s and allowing for measurement of around 1 adduct in 10^{10} unmodified nucleotides. It involves modification of the DNA after the formation of damage products with a tracer ^{32}P-labeled phosphate on modified nucleosides.[32,33] This technique involves four steps: First, DNA is enzymatically digested. Second, the DNA adduct is enriched by solvent or solid-phase extraction, immunoaffinity chromatography, HPLC, or further digestion. Third, the 5′-position of the adduct is labeled by polynucleotide kinase-mediated transfer of ^{32}P-orthophosphate from [γ-^{32}P]ATP, and fourth, separation is achieved by thin-layer chromatography or by HPLC. Adduct detection and quantitation are based on ^{32}P-decay.[29] This process requires about 1−10 μg of DNA.[32−34] Postlabeling is characterized by high versatility and screening capabilities, and has proven efficient for detecting adducts from complex mixtures such as cigarette smoke and environmental pollutants.[35−38] A major drawback limiting widespread clinical use is its lack of specificity and poor resolving capacity for distinguishing among different adducts. It also has drawbacks related with the sample preparation being labor intensive, and requiring the use and subsequent disposal of high levels of radioactivity. Additionally, there is the concern that false negative results result from failure to detect deglycosylated adducts.[21,29]

1.3.2. Immunoassay. Antisera elicited against chemically modified DNA have been used for quantitation or semi-quantitation, localization in nuclei of cells and tissues, and separation of a specific class of DNA adducts.[39−41] For example, an antiserum specific for the cisplatin-induced intrastrand bidentate adducts (platinum-GG and platinum-GA) has been used for the detection of cisplatin-adducts in white blood cells (WBCs) from testicular and ovarian cancer patients.[42−49] In another study, antibodies against dinucleotides containing platinum-GG or -GA adducts were used in a

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Table 2. Main Strategies Used for DNA Adduct Detection and Quantitation

method	sensitivity (adducts/nucleotide)	DNA required (μg)	advantages	limitations
radiolabeling	~1/10^9		sensitivity, straightforward determination	labeled compound needed, loss of isotope during metabolism or adduct formation, indirect measurement (radioactivity could occur by metabolic incorporation), only stable and exogenous adducts detected, high cost
^{32}P-postlabeling	~1/10^{10}	1−10	sensitivity, versatility, screening possible	high levels of radioactivity, no structural information, false negative due to loss of adducts, labor intensive
immunoassays	~1/10^8	1−200	adduct localization, cost, simplicity	antibody availability, cross-reactivity, relative quantitation, only stable adducts detected
MS	~1/10^8	10−100	very high specificity, structural information	adduct standard required for quantitation
AMS	~1/10^{12}	1−2,000	very high sensitivity, precision, and specificity	labeled compound needed, instrument availability, high cost, no information about nature or chemical form of isotope, indirect measurement (radioactivity could occur by metabolic incorporation), contamination with RNA-, protein-adducts, or unbound metabolite can be an issue

competitive enzyme linked immunosorbent assay (ELISA) after enzyme digestion of DNA and chromatographic separation of platinated dinucleotides.[50−52] With 1 adduct every 10^8 nucleotides, immunoassay techniques are useful tools with sufficient sensitivity for quantitation of clinically relevant levels of DNA modification;[53−55] however, their sensitivity is generally low compared to radio- or ^{32}P-postlabeling. A major advantage of antibodies is that their application is simple compared to more laborious techniques such as ^{32}P-postlabeling, and does not require specialized and expensive instrumentation such as mass spectrometry. However, detection of DNA adducts by immunoassays is limited by the effort, time, and cost for the generation of DNA adduct-specific antibodies,[21,29] by the cross-reactivity and nonlinear analytical responses, as well as by the large amounts of DNA necessary for each analysis.[6]

1.3.3. Mass Spectrometry. Mass spectrometry (MS) as a technique for DNA adduct analysis has unparalleled selectivity for chemical structures, with the process of repeated fragmentation of the parent ion in the context of tandem MS allowing one to derive direct information regarding DNA adduct structures. Sensitivity is in the range of 1 adduct in 10^8 nucleotides and is increased by coupling mass spectrometry with high performance liquid chromatography (HPLC), and by using modern ionization methods, such as electrospray ionization (ESI). The use of MS is limited by matrix effects caused by salts and other molecules that might be present in the samples, which can interfere with ionization or DNA adduct detection and quantitation. Extensive sample preparation steps can be involved in minimizing matrix effects, including enzyme hydrolysis and DNA adduct enrichment usually performed by solid-phase extraction. Additionally, quantitation of DNA adducts is most rigorously accomplished using stable isotope-labeled internal standards, which account for sample losses during sample preparation and ion suppression during MS analysis. Depending on the structure of the adduct, the synthesis of an isotope-labeled internal standard can be laborious and cost intensive. DNA adduct detection and quantitation by mass spectrometry and coupled techniques have been extensively reviewed previously.[19,20,56] Clearly, MS is increasingly capable of providing useful quantitation and structural information for clinical DNA adduct studies,[57−59] but it is not yet a viable platform for measuring drug−DNA adducts as biomarkers for clinical diagnostic applications. In order to be used in clinical diagnostics, limit of quantifications (LOQs) of greater than one adduct per 10^8 nucleotides are likely required.[6]

Inductively coupled plasma MS (ICP-MS) is a sensitive type of atomic mass spectrometry that reports on the amount of metals in a sample.[60−63] Compared to the methods described above, ICP-MS has high throughput with less tedious procedures and may be amenable to clinical studies.[64] Its ability to routinely measure adducts in the 1 per 10^8 nucleotide range from a few micrograms of DNA is compelling, even if structural information is limited.[65−68]

Accelerator mass spectrometry (AMS) is used to identify and quantify with high specificity, sensitivity, and precision rare, long-lived isotopes with attomole (amol) (10^{-18}) sensitivity for such labeled drugs and toxicants.[69,70] The principle of AMS relies in breaking down molecules into atoms that are then identified and quantified in a small particle accelerator. Labeling the sample with an isotope such as ^{14}C allows one to derive the concentration of the radiocarbon atoms in the particle beam

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Table 3. *In Vitro* Preclinical Evidence of Studies Investigating the Correlation between DNA Adducts Induced by Anticancer Drugs and Response[a]

treatment	biological model	DNA adduct detection method	response	year	ref
cisplatin	cell line(s)	HPLC-ICP-MS	sensitivity and resistance	2010	59
	cell line(s)	AAS	resistance	2000	66
	tumor biopsies	alkaline comet assay	cytotoxicity (FDA)	2009	27
	xenografts and tumor biopsies	^{32}P-postlabeling	animal and clinical response	1999	167
	cell line(s)	^{32}P-postlabeling	growth inhibition (SRB assay)	1999	168
	cell line(s)	AAS	cytotoxicity (MTT assay)	1997	169
	cell line(s), human buccal cells, lymphocytes, biopsies	ICC, IHC, and double-fluorescence microscopy	sensitivity and resistance	1997	170
	WBCs	ICP-MS	clinical response and toxicity	1996	64
	cells from bone marrow aspirates	ELISA	tumor remission	1994	171
	cell line(s)	RAGE and HPLC	cytotoxicity (MTT assay)	1994	172
	cell line(s)	IHC	clonogenicity (colony forming assay)	1991	173
	cell line(s)	alkaline elution, ELISA	clonogenicity (colony forming assay)	1991	99
	WBCs	ELISA	clinical response	1990	50
	cell line(s)	ICC	clonogenicity (colony forming assay)	1990	174
	cell line(s)	ICC	clonogenicity (colony forming assay)	1988	175
	cell line(s)	alkaline elution	clonogenicity (colony forming assay)	1984	176
	cell line(s)	alkaline elution	clonogenicity (colony forming assay)	1982	100
	cell line(s)	alkaline elution	growth inhibition (rel. cell number)	1981	177
	cell line(s) and xenografts	alkaline elution	clonogenicity (colony forming assay)	1981	102
cisplatin in combination with other drugs	cell line(s)	ICP-MS	growth inhibition (crystal violet stain)	2015	63
	circulating tumor cells in peripheral blood	IHC	clinical response	2013	65
	lymphocytes	alkaline comet assay	clinical response	2006	26
	lymphocytes	HCR assay (repair capacity)	clinical response	2002	166
	WBCs	ICP-MS	clinical response and toxicity	1996	64
carboplatin	cell line(s)	AMS	cytotoxicity (MTT assay)	2011	93
	tumor biopsies	alkaline comet assay	cytotoxicity (FDA)	2009	27
	cell line(s)	ICC	clonogenicity (colony forming assay)	1991	178
carboplatin in combination with other drugs	cell line(s)	AMS	cytotoxicity (MTS assay)	2015	90
	cell line(s)	ICP-MS	growth inhibition (crystal violet stain assay)	2015	63
	circulating tumor cells in peripheral blood	IHC	clinical response	2013	53
	lymphocytes	ELISA	*In vivo* vs *in vitro* adduct correlation	2001	87
oxaliplatin	cell line(s)	AMS	cytotoxicity (MTT assay)	2016	92
	cell line(s)	HPLC-AMS	sensitivity and resistance	2007	94
oxaliplatin in combination with other drugs	cell line(s)	ICP-MS	growth inhibition (crystal violet stain assay)	2015	63
diamminetetradichloroplatin	cell line(s)	alkaline elution	clonogenicity (colony forming assay)	1984	176
melphalan	cell line(s)	IHC	cytotoxicity (MTT assay)	2004	101
	cell line(s)	alkaline elution	clonogenicity (colony forming assay)	1991	99
	lymphocytes	ethidium bromide fluorescence assay	clinical response	1988	103
	cell line(s)	alkaline elution	clonogenicity (colony forming assay)	1987	98
	cell line(s)	alkaline elution	clonogenicity (colony forming assay)	1982	100
	cell line(s) and xenografts	alkaline elution	clonogenicity (colony forming assay)	1981	102
mechlor-ethamine	cell line(s)	alkaline elution	clonogenicity (colony forming assay)	1991	99
	cell line(s)	alkaline elution	clonogenicity (colony forming assay)	1987	98
PR104A	cell line(s)	nanoLC-ESI-MS	cytotoxicity (CellTiter Glo)	2017	190
	cell line(s)	alkaline comet assay	clonogenicity (colony forming assay)	2009	122
acylfulvene	cell line(s)	HPLC-ESI-MS	cytotoxicity (MTS assay)	2013	141
	cell line(s)	HPLC-ESI-MS	cytotoxicity (CellTiter 96 AQueous One assay)	2013	147
MC	cell line(s)	QPCR	cytotoxicity (MTT assay)	2010	179
	cell line(s)	immunofluorescence (phosphorylation of γH2AX)	cytotoxicity (MTT assay)	2007	180
	cell line(s)	HPLC-UV, LC-ESI/MS	clonogenicity (colony forming assay)	2002	132
	cell line(s)	HPLC-UV	clonogenicity (colony forming assay)	2001	137
	cell line(s)	alkaline elution	clonogenicity (colony forming assay)	1986	134
DMC	cell line(s)	QPCR	cytotoxicity (MTT assay)	2010	179

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Chem. Res. Toxicol. 2017, 30, 388−409

Table 3. continued

treatment	biological model	DNA adduct detection method	response	year	ref
	cell line(s)	immunofluorescence (phosphorylation of γH2AX)	cytotoxicity (MTT assay)	2007	180
	cell line(s)	HPLC-UV, LC-ESI/MS	clonogenicity (colony forming assay)	2002	132
doxorubicin	cell line(s)	radiolabeling and alkaline comet assay	apoptosis (flow cytometry)	2006	157
	cell line(s)	alkaline comet assay	cytotoxicity (growth inhibition assay)	2000	164
doxorubicin in combination with other drugs	cell line(s)	radiolabeling and alkaline comet assay	apoptosis (flow cytometry)	2008	151
	lymphocytes	alkaline comet assay	clinical response	2006	26
porfiromycin	cell line(s)	alkaline elution	clonogenicity (colony forming assay)	1986	134
fotemustine analogues	cell line(s)	alkaline comet assay	cytotoxicity (neutral red), clonogenicity (colony forming assay), and apoptosis (ELISA)	2003	181
temozolomide in combination with other drugs	cell line(s)	HPLC-MS	growth inhibition (SRB assay)	2009	182
S23906-1	cell line(s)	electrophoresis, fluorescence	cytotoxicity (CellTiter 96_ Aqueous assay)	2003	152
acronycine analogues	cell line(s)	electrophoresis, fluorescence	cytotoxicity (MTA assay)	2003	153
tirapazamine	xenografts	alkaline comet assay	response in xenografts	1997	115
	cell line(s)	alkaline comet assay	clonogenicity (colony forming assay)	1996	114
gemcitabine	cell line(s)	AMS	cytotoxicity (MTS assay)	2016	97

[a]AAS, atom absorption spectroscopy; AMS, accelerator mass spectrometry; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FACS, fluorescence-activated cell sorting; FDA, fluorescein diacetate assay; HPLC, high performance liquid chromatography; ICC, immunocytochemistry, ICP, inductively coupled plasma; IHC, immunohistochemistry; LC, liquid chromatography; MS, mass spectrometry; MTA, microculture tetrazolium assay; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; QPCR, quantitative polymerase chain reaction; RAGE, rotating field gel electrophoresis, SRB, sulforhodamine B; WBCs, white blood cells; γH2AX, gamma phosphorylation of histone H2AX.

and to estimate the concentration of drug in bodily fluids (e.g., whole blood, plasma, urine, or saliva), tissue, cells, protein, and DNA. Prior to AMS analysis, the sample usually has to be converted to graphite.[6] It is currently the most sensitive technique available for DNA adduct analysis.[71−73] Limitations of AMS are related to the requirement for using an isotope-labeled compound and specialized instrumentation. Contamination with RNA- or protein-adducts, unbound metabolite, as well as false positive events due to metabolic incorporation instead of DNA adduct formation should be carefully addressed in order to avoid an overestimation of DNA damage amounts.

1.4. Overview of Preclinical and Clinical Evidence of DNA Adducts Related with Response. The formation of DNA adducts has been addressed extensively in the context of initiation of carcinogenesis by genotoxins, such as from occupational, environmental, and dietary chemicals, and use as mechanism-based chemical biomarkers of genotoxin exposure.[74] Characterization of the same chemical process, in the context of DNA adduct formation from alkylating anticancer drugs is thus emerging as an indicator of responsiveness to therapy by cancer patients in the context of precision medicine. The focus of this perspective is on data pertaining to DNA damaging drugs for which the relationship between DNA adducts and cellular or patient responses has been characterized. Published studies concerning platinum-based drugs, nitrogen mustards, hypoxia-activated prodrugs, reductase-activated drugs, and minor-groove binding agents (Table 3 and Table 4) are thus presented and discussed with an emphasis on the nature of the predictive relationship obtained for biological responses as well as the bioanalytical methods used for adduct detection. For the drugs temozolomide, fotemustine, and gemcitabine, to our knowledge there has been only a single study reported for each concerning the relationship of DNA adducts with cytotoxicity, and these are summarized in Table 3 but not elaborated on further in the text. There are many other important DNA alkylating drugs such as nitrosoureas or alkylsulfonates that are not reported in this perspective because of a lack of data concerning the quantitative relationship between adduct levels with sensitivity in cancer cells or patients.

2. PLATINUM DRUGS AND COMBINATION THERAPIES

Platinum-based drugs, such as cisplatin, carboplatin, and oxaliplatin (Figure 2) are a cornerstone of modern combinatorial chemotherapeutic therapy and are used in over half of all chemotherapy patients for various cancers, including ovarian, breast, testicular, lung, colon, and bladder cancers, as well as lymphomas, myelomas, and melanomas.[75,76] Except in testicular cancer, their efficacy is limited by severe side effects and intrinsic or acquired drug resistance, eventually causing treatment failure.[75,77−79] The main mechanism of action of platinum drugs is to form covalent drug−DNA adducts, which interfere with transcription and replication to ultimately induce cell death via apoptosis or necrosis. The compounds predominantly react with guanine, and to a lesser extent, adenine nucleotides at the 7 position to form mono- (Pt-X) and diadducts (X-Pt-X or Pt-XX). The major diadduct product is an intrastrand cross-link (Pt-GG or Pt-GA), which is thought to invoke biochemical responses in cells by distorting the DNA helical structure. Cisplatin and carboplatin form structurally identical diadduct cross-links, which unfortunately leads to a similar resistance spectrum with these platinum drugs. However, oxaliplatin, a third generation platinum drug, has been shown to be active in cisplatin and carboplatin resistant cancers.[76]

Despite substantial understanding of the molecular mechanisms involved in drug resistance, little progress has been made in the prediction of cancer patient response to cytotoxic

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Chem. Res. Toxicol. 2017, 30, 388−409

Table 4. *In Vivo* Clinical Evidence of the Correlation between DNA Adducts Induced by Anticancer Drugs and Response[a]

treatment	biological model	DNA adduct detection method	response	year	ref
cisplatin	lymphocytes	HCR assay (repair capacity)	clinical response	2011	83
	xenografts	^{32}P-postlabeling	animal and clinical response	1999	167
	xenografts	^{32}P-postlabeling	GDF and T/C value	1999	168
	blood samples		clinical response	1998	54
	human buccal cells, tumor biopsies, paraffin-embedded tumor cells	ICC, IHC, and double-fluorescence microscopy	sensitivity and resistance	1997	170
	WBCs	ICP-MS	clinical response	1996	64
	several tissues	AAS and ELISA	tumor remission	1993	80
	WBCs	ELISA	clinical response	1990	55
	rats	ICC	histological alterations	1987	183
cisplatin in combination with radiation	normal (WBCs + buccal) and tumor biopsies	^{32}P-postlabeling	in tumor vs WBCs	2008	184
	primary tumor, WBCs, and buccal cells	^{32}P-postlabeling	clinical response	2006	82
	buccal mucosa		survival rate	2000	185
	buccal cells	ICC	clinical response	2000	85
	WBCs	AAS	clinical response	1996	186
cisplatin in combination with carboplatin	WBCs	AAS	clinical response	1993	49
	WBCs	AAS	resistance and toxicity	1993	187
	buccal cells	ICC	clinical response	1993	188
	WBCs	AAS	clinical response	1992	58
	WBCs	AAS	clinical response	1991	189
cisplatin in combination with other drugs	lymphocytes	alkaline comet assay	clinical response	2006	26
	WBCs	AAS	clinical response	1996	186
	WBCs	AAS	clinical response	1996	88
	WBCs	ICP-MS	clinical response	1996	64
	WBCs	ELISA, AAS	clinical response	1994	89
	blood cells	ELISA and AAS	clinical response	1993	47
	WBCs	ELISA	clinical response	1990	50
	blood cells	ELISA	clinical response	1988	44
	WBCs	ELISA	clinical response	1987	45
	blood cells	ELISA	clinical response	1986	48
	blood cells	ELISA	clinical response	1985	46
carboplatin	tumor biopsies and blood cells	ICP-MS	clinical response	2012	65
	lymphocytes	HCR (repair capacity)	clinical response	2011	83
	WBCs	ICP-MS	clinical response	1996	64
	several tissues	AAS and ELISA	tumor remission	1993	80
	WBCs	ELISA	clinical response	1990	55
	rats	ICC	histological alterations	1987	183
carboplatin in combination with other drugs	blood cells	AMS	clinical response and animal survival	2016	91
	tumor biopsies and blood cells	ICP-MS	clinical response	2012	65
	mononuclear cells from bone marrow aspirates	ICP-MS	bone marrow response, and clinical response	2004	57
	WBCs	ELISA, AAS	clinical response	1994	89
	blood cells	ELISA and AAS	clinical response	1993	47
oxaliplatin	lymphocytes	HCR (repair capacity)	clinical response	2011	83
oxaliplatin in combination with other drugs	WBCs	adsorptive stripping voltammetry	clinical response	2008	86
cyclophosphamide in combination with other drugs	blood cells	alkaline elution	clinical response	1992	105
	blood cells	HPLC-LSI-MS/MS	clinical response	2012	106
melphalan	WBCs	gel electrophoresis and Southern blotting	clinical response	2003	104
doxorubicin in combination with other drugs	lymphocytes	alkaline comet assay	clinical response	2006	26
	lymphocytes	alkaline comet assay	clinical response	2000	164

[a]AAS, atom absorption spectroscopy; ELISA, enzyme-linked immunosorbent assay; GDF, growth delay factor; HCR, host-cell reactivation; HPLC, high performance liquid chromatography; ICC, immunocytochemistry; ICP, inductively coupled plasma; IHC, immunohistochemistry; LSI, laser spray ionization; MS, mass spectrometry; T/C, treated/control; WBCs, white blood cells.

chemotherapy drugs in clinical practice. Currently, most platinum-based therapeutic strategies are one-size-fits-all and trial-and-error approaches whereby they are administered at high therapeutic doses in combination with one or more other drugs followed by monitoring of response over relatively long periods of time (Figure 1).[6]

Over the last three decades, the relationship between platinum drug-induced DNA adduct levels with cytotoxicity

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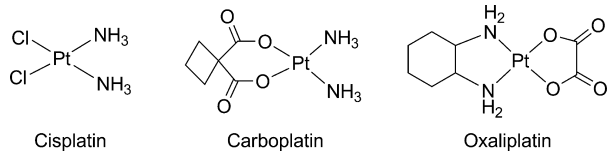

Figure 2. Platinum drugs.

has been addressed in a number of preclinical and clinical studies.[42,46,47,80−87] For example, in a series of ELISA-based clinical studies of ovarian and testicular cancer patients conducted by Reed et al.,[44,45,48,81] significantly higher platinum-DNA adduct levels were observed in isolated leukocyte DNA after cisplatin- or carboplatin-based chemotherapy in patients with good clinical outcome. They further expanded the patient population to include breast, lung, colon, and squamous sarcoma cancer patients, by analyzing platinum levels in leucocyte DNA via AAS.[49,80] More recently, Pieck et al. investigated the formation of oxaliplatin-DNA adducts in WBCs isolated from patients with various malignancies.[86] The use of the more sensitive adsorptive stripping voltammetry enabled the quantitation of the generally lower oxaliplatin-DNA adduct level after administration of systemic oxaliplatin based chemotherapy. There were significantly higher platinum−nucleotide ratios at 24 and 48 h after the start of infusion in responders compared to that in nonresponders.[86]

There are conflicting results concerning the relationship of DNA damage by platinum drugs and responses, depending on whether two different detection methods, ELISA or AAS, were correlated to clinical outcome.[47,80] Low DNA adduct levels measured by ELISA consistently correlated with disease progression, whereas in some initial AAS-based studies, the incorporation of total platinum failed to show such a correlation.[64,88,89] Adduct level measurements via ELISA and AAS by Motzer et al., or via ICP-MS by Bonetti et al., showed no differences between responsive and nonresponsive patient populations.[64,89] There are several possible reasons for such conflicting data including differences in the sensitivity of various tumor types to platinum based therapy, drug regimes, study designs, analytical detection methods employed and small numbers of patients. A likely reason for the data discrepancy could be variations in experimental procedures used by different investigators. For example, Blommaert et al. used ICC nuclear stain accumulation of buccal mucosa cells as a surrogate for WBCs or tumor tissues and observed a moderate correlation of carboplatin-adduct formation and good clinical response, whereas the cisplatin nuclear stain failed to correlate with treatment response.[95] The ICP-MS-based study published by Bonetti et al.[64] had a 24% incidence of samples below the LOD and failed to show a correlation of platinum adduct level and response. Nel et al. studied the formation and repair of Pt-(GpG) intrastrand cross-linked DNA adducts in isolated CTCs.[53] They treated and immunostained cells from patients with advanced non-small-cell lung cancer (NSCLC) *ex vivo* with cisplatin prior to patient systemic therapy. Platinum-DNA adduct formation (curve max, area under the curve (AUC), and curve slope (repair)) correlated significantly with patient response to subsequent platinum based chemotherapy.[53]

A major concern in the interpretation of adduct data is the possibility that, in clinical practice, the majority of patients are being treated with one or more additional anticancer drugs, to induce synergistic effects and to enhance efficacy, and that depending on tumor type, the nonplatinum combination drugs could contribute more to the overall response. These combination therapies can include antimitotics, chain terminating nucleoside analogues, topoisomerase inhibitors, angiogenesis inhibitors, or more recently, the molecular targeted therapies (e.g., paclitaxel, gemcitabine, doxorubicin, everolimus, or gefitinib, respectively). In a recent ICP-MS based preclinical study, Chen et al. showed that the combination of D-penicillamine with oxaliplatin or cisplatin led to an increase in platinum-adduct formation and cytotoxicity in the resistant cervical carcinoma cell line.[63] Similarly, Jiang et al. reported increased carboplatin-DNA adduct formation and cytotoxicity in an urothelial carcinoma cell line when cotreated with paclitaxel.[90] However, the *in vivo* contribution of each individual drug in a regimen to tumor response is more complicated to delineate than in cell culture cytotoxicity experiments. In a recent report by Zimmermann et al., gemcitabine- and carboplatin-DNA adduct levels were shown to correlate with response to chemotherapy in four patient derived xenograft (NSG-PDX) mouse models of bladder cancer.[91] In one of the PDX models, the drug−DNA adduct levels were influenced by the combination drug, and this model had an apparent synergistic response (enhanced normalized tumor volume reduction) to the combination treatment. In a report by Cooper et al., leukemia patients were administered carboplatin, fludarabine, and escalating doses of topotecan, and no correlation of a small patient cohort's response with carboplatin DNA adduct formation at 48 h was observed, possibly due to the complex three drug clinical strategy and measurement of only one pharmacodynamic end point. However, patients with faster reduction of adduct levels (DNA repair) had a higher chance of relapse.[57] Despite the previous contradictory conclusions, drug−DNA adduct levels as pharmacodynamic end points remain potentially useful as indicators of drug response to cytotoxic chemotherapy agents.

Because of the insufficient sensitivity of available standard detection methods (i.e., ICP-MS, immunoassays), platinum-DNA adduct levels are commonly measured only after exposure to high, therapeutically relevant drug concentrations. An *in vivo* diagnostic microdosing strategy is being perused by Henderson and co-workers, by a combination of microdosing with a ^{14}C labeled drug and adduct determination of tissue samples (PBMCs or tumor biopsy) via AMS prior to chemotherapy. AMS is sufficiently sensitive to enable the study of platinum drug DNA adduct formation at subtherapeutic levels, termed a diagnostic microdose, which has the potential to predict response to treatment.[91−97] The investigators have demonstrated proof of principle of this approach through two model systems: (1) cell culture studies using multiple cancer lines[92,96,97] and (2) mouse tumor xenografts.[93] Three key observations were made: (a) the level of drug−DNA adducts is low in resistant cancer cells and high in sensitive cancer cells; (b) diagnostic microdosing predicts the level of therapeutic-induced carboplatin- or oxaliplatin-DNA adducts; and (c) microdose-induced carboplatin-DNA monoadduct frequencies correlate with PDX tumor response toward full platinum based chemotherapy in mice. This work has evolved into two ongoing clinical studies in cancer patients to assess the predictive capabilities of this approach (Clinicaltrials.gov identifiers NCT01261299 and NCT02569723). Preliminary results recently reported show a strong predictive relationship between microdose-induced carboplatin-DNA monoadducts in PBMC's from bladder cancer patients and response to standard carboplatin- or cisplatin-based chemotherapy (Figure 3).

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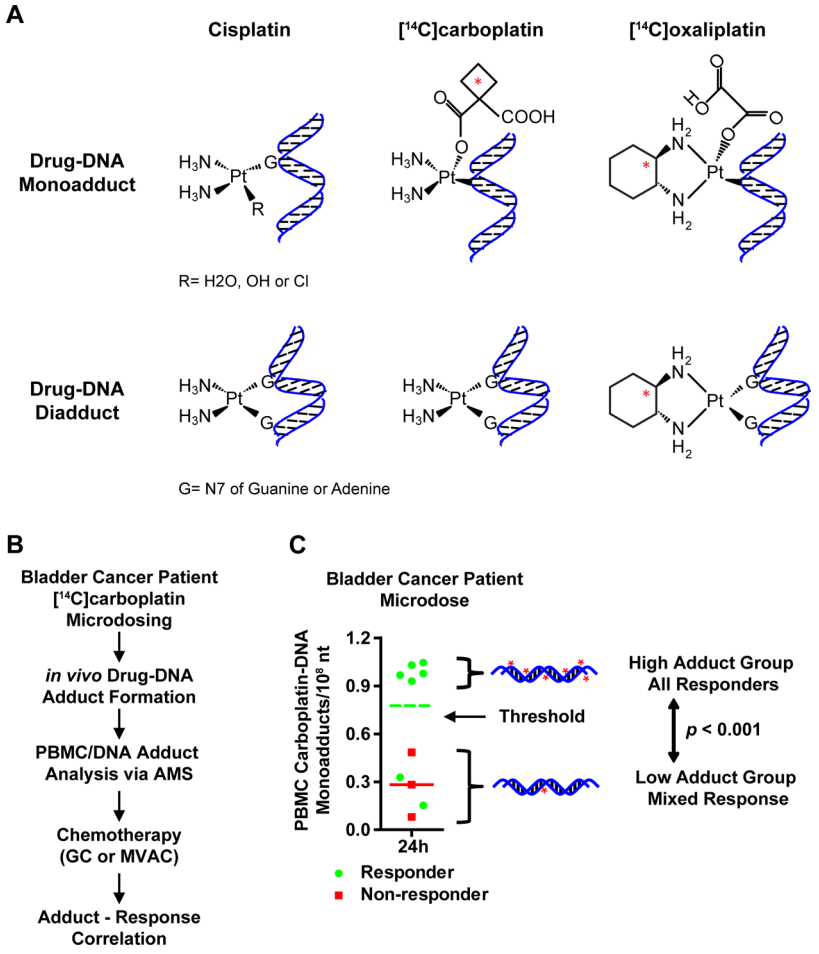

Figure 3. Diagnostic microdosing for the prediction of patient response. (A) Cisplatin, carboplatin, and oxaliplatin DNA adduct structures (* denotes the presence of a ^{14}C atom used in AMS studies). (B) Schema for developing carboplatin-DNA adducts as biomarkers of bladder cancer chemotherapy response. (C) Preliminary data from a clinical study using AMS analysis of PBMC from bladder cancer patients given [^{14}C]carboplatin diagnostic microdoses followed 24 h later by blood sampling and by subsequent platinum-based chemotherapy. The carboplatin-DNA adduct data were correlated with patient response to chemotherapy. GC, gemcitabine and cisplatin; MVAC, methotrexate, vinblastine, doxorubicin, and cisplatin.

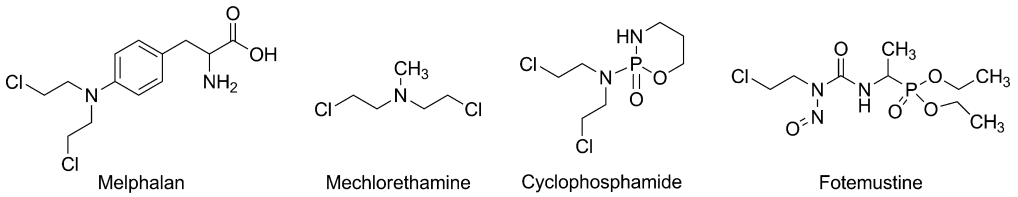

Figure 4. Nitrogen mustards and half mustard analogues.

3. NITROGEN MUSTARDS AND HALF-MUSTARDS

Nitrogen mustards were the first DNA alkylating drugs used in early cancer chemotherapy in the 1950s. Variants of these compounds are used in modern clinical therapy of leukemias and sarcomas, and of cancers of the breast, ovary, testes, and lung. Nitrogen mustards are bifunctional chemicals, inducing interstrand cross-linking of DNA typically by alkylating the 7-position of two guanines in GNC·GNC (5′ → 3′/5′ → 3′) sequences. Although the cross-links are considered to be the most severe form of alkylation damage to DNA and responsible for inducing cell death, it is typically the case that more modification occurs in the form of monoadducts from alkylation at various positions, such as 7- or 3-Gua.[9]

Mechlorethamine, an aliphatic nitrogen mustard, and melphalan, an aromatic nitrogen mustard, both induce guanine 7 mono and cross-linked adducts, and melphalan has been shown to also react with adenine at the 3 position (Figure 4).[9] In a series of studies reported by Hansson et al., the relationship of DNA adduct formation and removal with cellular response was evaluated for these two compounds in human melanoma cell lines.[98,99] Alkaline elution was used for DNA cross-linking analysis, and the formation of colonies was used as a basis for characterizing responsiveness to the drug. For both drugs, clonogenicity increased with increases in the total area under the curve for DNA interstrand cross-links (ICLs).[98,99] In a related *in vitro* study concerning three Burkitt's lymphoma cell lines, DNA interstrand cross-linking was

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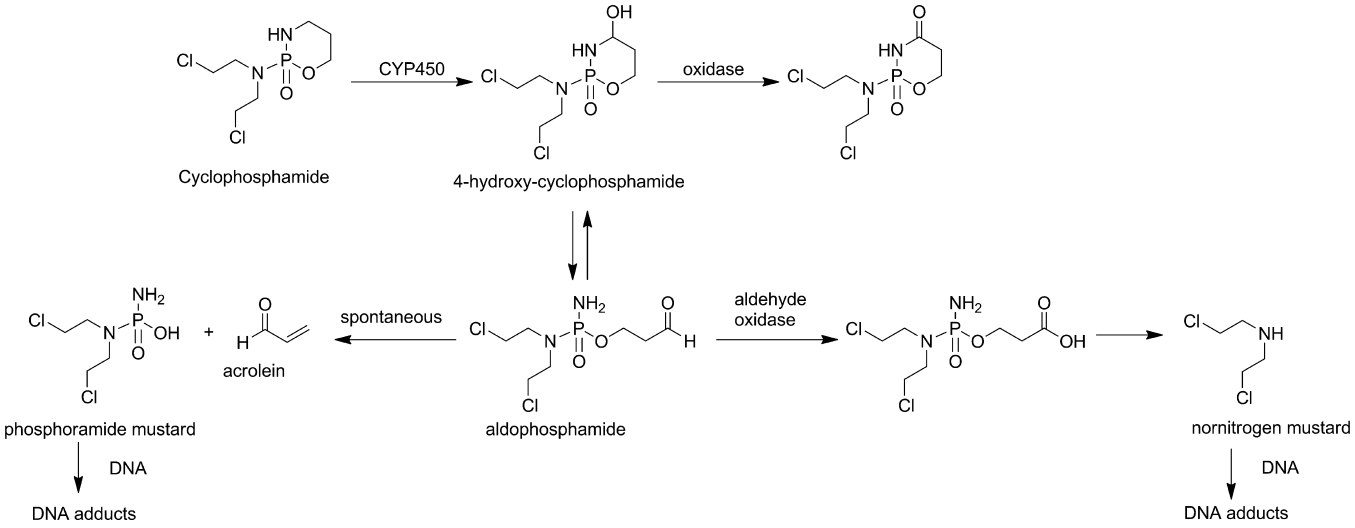

Figure 5. Cyclophosphamide and its mechanism of activation.[9]

measured by alkaline elution and compared to colony formation. ICLs increased with increasing dose and correlated with an increase in clonogenicity to melphalan treatment, also suggesting that DNA ICLs could be predictive of sensitivity.[100] Using the monoclonal antibody MP5/73 and immunohistochemistry (IHC) visualization, melphalan-specific DNA adducts were compared in three human colon cancer cell lines (HT-29, SW480, and SW1116) and one rat colon cancer cell line (CC531) exposed to increasing concentrations of melphalan. Increasing doses of melphalan led to increases in DNA adduct staining intensity and reduced cell metabolic activity (MTT assay).[101] In another study, the relationship between DNA cross-linking, determined by alkaline elution, and cell death induced by melphalan in a drug-resistant L1210 leukemia cell line, indicated that interstrand cross-linking was reduced in the resistant cell line relative to the parent line, with cytotoxicity in the resistant line being reduced by approximately the same proportion as the cross-links.[102] Furthermore, the relationship between DNA cross-linking and cell killing was assessed in mice bearing the sensitive and resistant L1210 leukemia cell lines. The different cells were isolated from mice and treated *in vitro* with melphalan (20 μM for 1 h), and DNA cross-linking was determined.[102] The melphalan-resistant cells exhibited marked reductions of cross-linking which correlated with a reduced survival rate compared to the corresponding parent cells.[102]

In clinical studies aiming to determine if DNA cross-linking by melphalan is predictive of sensitivity, relationships among transport, metabolism, and DNA interactions of melphalan have been addressed in lymphocytes from patients with chronic lymphocytic leukemia.[103] Using an ethidium bromide fluorescence assay to monitor DNA alkylation, it was found that lymphocytes that were removed from untreated patients and then treated with 5 μM melphalan had a statistically significantly greater percentage of cross-links than in lymphocytes derived from resistant patients.[103] Finally, melphalan-induced DNA mono- and cross-linked adducts located in *TP53* and *N-ras* gene sequences of genomic DNA from human peripheral lymphocytes of multiple myeloma patients following *in vitro* exposure or *in vivo* treatment to melphalan have been characterized.[104] Of seven patients with multiple myeloma treated with high dose melphalan, three with the lowest levels of DNA damage in *TP53* and *N-ras* did not respond to melphalan therapy.[104] These studies support that DNA adducts from melphalan, both in preclinical and clinical contexts, may be predictive of drug response.

The nitrogen mustard cyclophosphamide (Figure 4), in contrast to melphalan and mechlorethamine, requires metabolic activation by CYP2B6 to the phosphoramide mustard in order to form DNA adducts (Figure 5).[9,105,106] In peripheral mononuclear blood cells (PBCs) of 15 ovarian carcinoma patients receiving a therapy regime which combined cyclophosphamide and carboplatin, DNA strand breaks and cross-links measured by alkaline elution had considerably increased mean elution rates for treated patients when compared to that of healthy controls. A strong increase in elution rate was considered possibly linked to therapeutic success when comparing six patients at the same stage of cancer, but the authors were cautious to draw such conclusions due to the low number of patients.[105] In another study, the interstrand DNA cross-link G-NOR-G was quantified by HPLC-MS/MS in blood cells of Fanconi anemia (FA) and non-FA cancer patients receiving cyclophosphamide-based therapy prior to hematopoietic cell transplantation (HCT).[106] Quantitation of this adduct revealed that FA patients produced 15-fold higher adduct levels than non-FA patients, confirming the hypothesis that FA patients are hypersensitive to DNA alkylating agents and require a lower dose of cyclophosphamide during treatment.[106] This FA study was the first instance of low abundant cytotoxic G-NOR-G adducts being measured in blood cells, underlying the sensitivity of HPLC-MS/MS approaches in the assessment of DNA adducts by DNA alkylating drugs in samples derived from patients.[106] Furthermore, this example involves a nitrogen mustard drug that requires metabolic activation, and there appears to be a predictive relationship between DNA adducts and response since metabolic activation is taken into account with the adduct measurement.

4. HYPOXIA-ACTIVATED PRODRUGS

Tumor hypoxia, a state of low oxygen concentration common in tumor cells, is associated with radio- and chemotherapy resistance. Nonetheless, it also provides an opportunity for selective targeting of tumor cells with hypoxia-specific gene

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therapy, recombinant anaerobic bacteria, and hypoxia-activated prodrugs (HAPs). HAPs are preferentially activated in hypoxic cells by enzymatic reduction, for example, of nitro groups, quinones, aromatic *N*-oxides, aliphatic *N*-oxides, and transition metals. A general chemical mechanism for activation of HAPs involves the formation of free radical intermediates by one-electron reductases and further reduction to cytotoxic drugs in hypoxic cells. The formation of the initial free radical intermediates is inhibited in oxygenated cells, where the radical intermediates are reoxidized to the original prodrug (Figure 6).

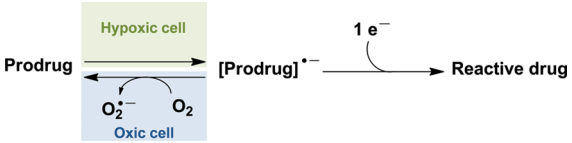

Figure 6. Mechanism of activation of hypoxia-activated prodrugs.

Hypoxia, as well as expression levels and diversity of functional one-reductase enzymes, determine HAP selectivity for tumor vs normal cells, and these factors can be monitored in tumor samples to predict HAP potency. They can be divided into two groups: those activated under moderate hypoxia, such as tirapazamine, or those activated under severe hypoxia, such as PR104 (Figure 7). In the second case, it is essential that the

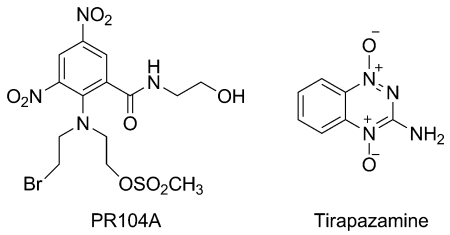

Figure 7. Hypoxia-activated prodrugs.

active drug diffuses into less hypoxic regions of the cancer in order to also have an effect in cells that are only moderately hypoxic.[107−109]

Tirapazamine (TPZ, Figure 7) is an HAP from the class of the benzotriazine di-*N*-oxides designed more than 30 years ago. It was the first drug introduced into the clinic as a bioreductive agent. It has been shown to induce DNA single strand breaks (SSBs), double strand breaks (DSBs), and chromosome aberrations.[110,111] TPZ potentiates the effect of radiation therapy (RT) by killing hypoxic cells in the tumor, which are the most radiation-resistant.[109] TPZ has also been shown to enhance the efficacy of cisplatin by increasing its sensitivity rather than from a combined effect in cells.[109] The combination of TPZ with cisplatin has been tested in a Phase III clinical trial with advanced NSCLC, and was found to double the overall response rate and significantly prolong survival when compared to that of cisplatin standard treatment.[109,112] In a randomized Phase II study with 122 previously untreated patients with stage III/IV of head and neck cancer, the addition of TPZ to cisplatin-based chemoradiotherapy improved patient survival over cisplatin-based chemoradiotherapy treatment only.[113] In addition, a strong correlation between hypoxic cytotoxicity and TPZ-induced DNA SSBs measured by the alkaline comet assay has been found in human (A549, HT1080, and HT-29) and murine tumor cell lines (EMT6, RIF-1, and SCCVII).[114] The same cell lines were also subcutaneously transplanted in mice to investigate whether levels of DNA damage induced by TPZ can be used to predict the response to fractionated radiation.[115] The level of DNA damage, measured after a single TPZ dose, distinguished TPZ nonresponsive HT1080 tumors but could not predict the enhancement of radiation cell killing for the other tumor types. The authors hypothesized that the correlation between DNA damage and radiation potentiation previously observed in cell lines also exists in xenograft models but that it could have been masked by the large variation in the DNA damage measured between individual tumors.[115]

PR104 is an experimental hypoxia-activated DNA alkylating drug under investigation for the treatment of leukemias (Figure 7).[116,117] When administered to patients, PR104 is systemically hydrolyzed to the corresponding alcohol PR104A, which is metabolically activated to cytotoxic hydroxylamine (PR104H) and amine (PR104M) metabolites by one-electron reductases or by the two-electron reductase AKR1C3 in an oxygen-independent reaction.[118−121] PR104A induces DNA mono- and interstrand cross-links, mainly at the 7-position of guanine, and 7/3-position of adenine.[119,122,123] Recent cell-based studies concerning this drug have centered on better understanding properties of cancer cells that make them most responsive to PR104, as well as how cells can be activated to have increased susceptibility to the drug. Thus, when HT-29 colon cancer cells were preconditioned with a low dose of the isothiocyanate sulforaphane (SF), which alone had no appreciable influence on cell viability, enhanced the capacity of PR104A to induce cell death[124] and, furthermore, led to an increase in PR104A DNA adducts relative to nonpreconditioned controls. The analysis was carried out by LC-MS/MS; therefore, it could be ascertained that adducts whose levels increased were those with masses indicating that they were formed from PR104A metabolites. On the basis of previous data concerning the influence of SF-preconditioning on HT-29 cells, it can be concluded that the mechanism of increased metabolite-adduct formation results from increased levels and activity of the enzyme AKR1C3, which is the main reductase thought to activate PR104A under normoxic conditions.[190] The higher enzyme expression results in more extensive drug metabolism/activation and therefore higher levels of adducts in increased cytotoxicity in the target cells.

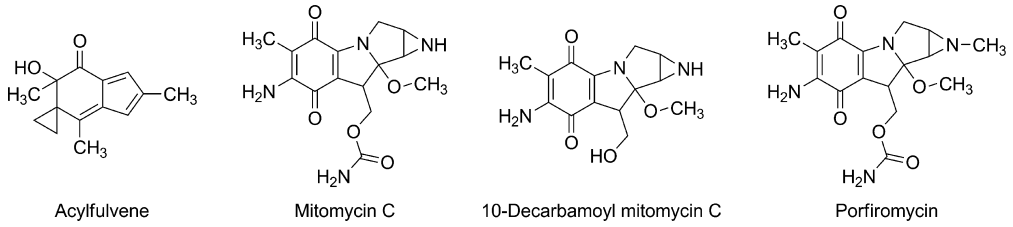

Figure 8. Reductase-activated drugs.

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Chem. Res. Toxicol. 2017, 30, 388−409

5. REDUCTASE-ACTIVATED DRUGS

Reductase-activated drugs rely on metabolic activation by an enzyme-catalyzed reduction for generating cytotoxic metabolites. The expression levels of bioreductive enzymes in tumor cells relative to normal tissues largely dictate sensitivity for this class of DNA alkylating agents. Tumor hypoxia may also enhance the sensitivity of cells to reductase-activated drugs, for example, by activation of the drug to the cytotoxic metabolites by one-electron reductases under hypoxic conditions. Thus, although PR104, discussed in the last section is classified as a HAP per its original design, it could also be considered a reductase-activated drug per the discovery of the role of AKR1C3 reduction in its activation.[121]

Mitomycins are antibiotics discovered in the 1950s and produced by the microorganism *Streptomyces caespitosus*.[125] The most investigated member of this family, mitomycin C (MC, Figure 8), additionally has anticancer activity and has been widely used in cancer chemotherapy for the treatment of various cancers such as breast, bladder, stomach, pancreas, lung, and liver. Upon reductive activation, MC alkylates DNA and induces at least six different DNA adducts, and among them a cross-link between two guanines alkylated at the N2 position.[126−128] Several synthetic and natural analogues of mitomycins have been studied for their anticancer properties and as mechanistic probes of biochemical processes that influence their activity. Among these analogues, the MC derivative 10-decarbamoyl mitomycin C (DMC, Figure 8), obtained by chemical removal of the 10-carbamoyl group of MC, was first synthesized to study structure−activity relationships[129] and was subsequently investigated for its capacity to alkylate DNA.[128,130−133]

The formation of DNA interstrand cross-links, measured by alkaline elution, correlated with cytotoxicity for MC in mouse mammary tumor EMT6 and Chinese hamster ovary (CHO) cells grown under hypoxia, with more being formed in these conditions than under anoxia.[134] In EMT6 cells, MC was more toxic than porfiromycin (PM) under normoxia, and the DNA ICL amounts observed for MC and PM under these conditions correlated with the cytotoxicity.[134] To test the hypothesis that MC-induced DNA adducts derive from two distinct pathways, namely, direct alkylation by MC or alkylation after bioreduction to the major reductive metabolite (2,7-DAM), the same cell line was used to characterize the relationship among reductive metabolism of MC, DNA adduct formation, and cytotoxicity. In fact, there were four DNA adducts identified as directly originating from MC, and two derived from the alkylation of 2,7-DAM.[135,136] When EMT6 cells were treated with 2,7-DAM, the same adducts were observed as when the cells were treated with MC. However, 2,7-DAM has dramatically lower potency with regard to cytotoxicity, and results suggested that the corresponding adducts are not important for MC cytotoxicity.[135,137] It was hypothesized that the formation of the adducts by 2,7-DAM is a mechanism to inactivate MC, but if true, these results would be in disagreement with the hypothesis that DNA adducts by DNA alkylating drugs correlate with cytotoxicity. However, the same study also found a correlation between MC-derived DNA adducts and cytotoxicity by treating these cells with MC.[137]

The synthetic MC metabolite DMC (Figure 8) was found to be slightly more toxic than MC in a variety of cell lines, including repair-deficient variants.[138] To understand the basis of the reduced cytotoxicity of MC vs DMC, DNA adducts resulting from treatment of EMT6 cells with equimolar concentrations of DMC and MC were assessed.[132] DMC was found to induce both monofunctional and bifunctional adducts resulting from reactions with 2′-deoxyguanosine (dGuo) in genomic DNA, a pattern similar to that observed for MC. However, DMC induced 20−30 times more total adducts than MC in EMT6 cells. For example, for DMC and MC, the total adducts induced by 10 μM drug treatment were 5.4 adducts per 10^5 nucleotides and 0.26 adducts per 10^5 nucleotides, respectively. Surprisingly, the interstrand cross-linking activity of DMC in cells was found to be weaker compared to that of MC: DMC-induced cross-links represented only 7% of total adduct measured, whereas MC-induced cross-links represented 27%.[132] Furthermore, evaluation of colony forming assay data indicated that DMC was slightly more toxic than MC. Thus, for both molecules, cytotoxicity correlated with DNA cross-linking activities but not with their total monoadduct burden.[132]

Acylfulvenes (AFs) are another class of reductase-activated DNA alkylating drugs. They are derived from the natural product illudin S. These compounds are notable for reasonable selective toxicity to certain cancer cells, especially those with high expression of prostaglandin reductase 1 (PTGR1), which bioactivates the drug.[139−141] The activated intermediate of AF reacts with DNA resulting mainly in alkylation at the 3 position of adenine but also of 7- and 3-positions of guanine.[140] The adducts induced by the substituted AF analogue HMAF, which has been the most advanced clinical candidate among this class, as well as the original natural product illudin S, appear to be exclusively repaired by the transition-coupled subpathway of nucleotide excision repair (NER) pathway.[142−146]

Illudin S and acylfulvene (AF, Figure 8) have been used as a complementary pair of probes to better understand how drug cytotoxicity relates to cellular bioactivation capacity and DNA adduct formation for reductase-activated DNA alkylation because the structurally related compounds have very different cell selectivity profiles. To this purpose, a colon cancer cell line (SW-480) was engineered to stably overexpress PRGR1, the reductase enzyme required for activating AF. Cytotoxicity and DNA adduct levels quantified by LC-ESI-MS/MS were compared for the two compounds in the PTGR1-engineered and normal cell lines.[141] AF induced more DNA adducts and exhibited increased cytotoxicity in cells overexpressing PTGR1, whereas illudin S-induced DNA adducts also correlated with cytotoxicity, but these factors were not influenced by the levels of PTGR1 in the cells.[141] Additionally, it has been hypothesized that inhibiting the NER pathway would result in the persistence of AF-DNA adducts and therefore increase its cytotoxicity. Thus, HT-29 cells were treated with AF in combination with nontoxic doses of UCN-01, which prevents cells from undergoing NER, or methoxamine, a base excision repair (BER) inhibitor, as comparison. Both cytotoxicity and the major adducts were quantified using LC-ESI-MS/MS and 3-AF-Ade-d_3 as internal standard were measured.[147] Impairing NER function, but not BER, led to the persistence of AF-induced DNA adducts and promoted AF cytotoxicity by reducing the concentration required to kill HT-29 colon cancer cells by 2-fold.[147] This study also measured the formation and repair of AF-induced adducts versus time by exposing the cells to 500 nM AF for 24 h and then to fresh medium lacking the drug for 48 h. During the first 24 h, AF-induced adduct levels increased with increasing time of AF exposure, whereas a decrease in adduct levels was observed for the 48 h that followed medium replacement.[147] Studies addressing the

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Chem. Res. Toxicol. 2017, 30, 388−409

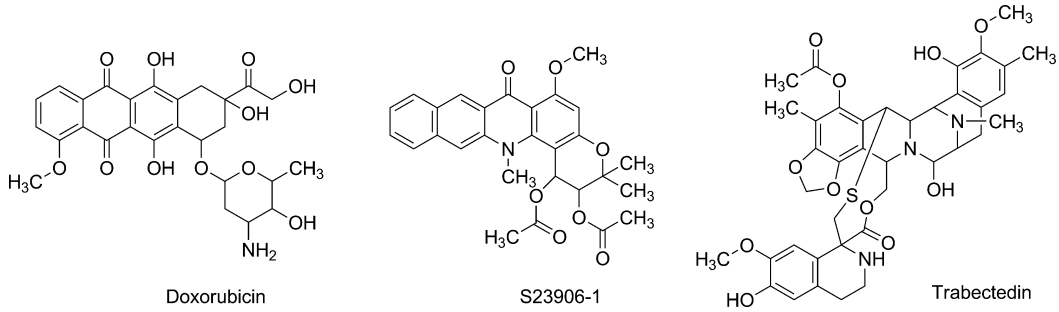

Figure 9. Minor groove binding agents.

kinetic of formation and repair of DNA adducts may be useful for identifying the appropriate timing of sample collection for diagnostics tests.

6. MINOR-GROOVE BINDING AGENTS

The spatial arrangement of base pairs in the DNA double helix gives rise to the major and minor grooves. With a width of 11.6 Å and depth of 8.5, the major groove is characterized by multiple sites of interaction for strong binding to drugs and ligands, and it provides easy access for large molecules. On the other hand, with a width of 6.0 Å and a depth of 8.2, the minor groove is smaller and has fewer binding sites available for binding interactions or reactions. However, the minor groove is accessible for reacting with small molecules such as antibiotic and anticancer drugs.[148,149] Doxorubicin (DOX), acronycine, and tetrabectedin are DNA minor-groove binding agents (Figure 9). Studies have been carried out to understand how the formation of DNA adducts from these three drugs relates to cellular responses in cell lines and in patient-derived cells.[26,150−154]

DOX is a chemotherapeutic agent belonging to the class of anthracyclines antibiotics, which also include daunorubicin, idarubicin, and epirubicin. It is used clinically, usually in a combination of drugs with a complementary mode of action, against breast, lung, thyroid, and ovary carcinomas, leukemias, and Hodgkin's and non-Hodgkin's lymphomas. DOX is classified as a topoisomerase II poison since it binds to DNA-associated enzymes, such as topoisomerase I and II, but it may perturb several other biochemical processes in affecting its anticancer activity. Inhibition of topoisomerase II results in DSBs, which if not repaired can initiate apoptosis.[155,156] It also forms covalent DNA adducts, and the toxicity of these lesions toward cells is higher than that induced by topoisomerase II impairment.[155,157] The resulting DNA adducts are predominantly induced at 5′-GC-3′ sites via reaction at the N2 amino group of dGuo.[158−162] Formaldehyde plays a central role in DNA adduct formation by DOX since it provides a methylene group that links the 3′-amino group of DOX to the 2-amino group of dGuo in DNA via Schiff base chemistry.[158,163] A peculiarity of these adducts is that they are formed by covalent binding to one DNA strand but are resistant to thermal denaturation due to the stabilization to the local region of DNA.[159]

The cytotoxicity of DOX-induced DNA adducts has been extensively addressed. In HL-60 cells, adducts induced by cotreatment with DOX and nontoxic doses of formaldehyde-releasing prodrugs increased cytotoxicity compared to the treatment with DOX only. Additionally, fewer topoisomerase II-mediated DSBs were observed by the combined treatment, suggesting that the presence of formaldehyde shifts the mode of action of DOX from inhibiting topoisomerase II to inducing DOX-DNA adducts.[157] The same authors investigated the effect of topoisomerase II inhibitors on DOX-induced topoisomerase II-mediated DSBs and on formaldehyde-mediated DOX-DNA adducts.[151] They found that the topoisomerase II inhibitor subuzoxane released formaldehyde and shifted the mechanism of DOX toxicity to the formation of DOX-DNA adducts, which enhanced the apoptotic response compared to DOX as a single treatment in HL-60 cells.[151]

The alkaline comet assay was used to assess DNA damage in peripheral blood lymphocytes (PBLs) of cancer patients receiving DOX-based treatment. In an attempt to develop a predictive test of outcome prior to chemotherapy, Nadin et al. determined individual influences of the *in vitro* exposure DOX and *in vivo* treatment with DOX-based regimes on DNA damage, DNA repair, and mismatch repair proteins hMSH2 and hMLH1 protein expression in PBLs isolated from 25 cancer patients pre- and postchemotherapy and related them to patient response.[26] The results were that DNA damage induced by *in vitro* treatment with DOX did not distinguish between patients showing complete response and those showing partial response, stable disease, or progressive disease.[26] The authors suggest that the inability to distinguish between patient response by means of DNA damage may be related to low nuclear expression of topoisomerase II, the main target of DOX. Similarly, Johnstone et al. also reported that levels of DOX-induced DNA damage, assessed by the alkaline comet assay, was not predictive of response in PBLs isolated from breast cancer patients *in vivo*, even though in the latter case an apparent difference could be observed between patients that relapsed and those that did not, but this difference was not statistically significant.[164] Nonetheless, in the same study a correlative relationship was observed between cytotoxicity and DNA damage in a human breast cancer cell lines *in vitro*.[164]

For another class of minor groove binding agents, the acronycines, there is data available concerning the relationship of DNA damage with responses of cell lines. For example, for the benzoacronycine derivative S23906-1 (Figure 9), a potent antitumor agent that alkylates mainly N2-Gua in DNA and for which activity is modulated by glutathione (GSH),[153,165] a correlation was observed between drug-induced DNA adducts and cytotoxicity. Elevated GSH levels in KB-3-1 epidermoid carcinoma cells were associated with a 3-fold reduction in cytotoxicity, which was attributed to a reduced formation of drug−DNA adducts. Additionally, treatment of cancer cells with buthionine sulfoximine, an inhibitor of GSH biosynthesis, led to an increase in the formation of S23906-1-DNA adducts and promoted cytotoxicity.[152] To elucidate the precise molecular mechanism of DNA alkylation by S23906-1 and to understand its involvement in cytotoxicity and antitumor

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properties, an evaluation of structure−activity relationships was performed with a series of structural analogues of S23906-1 in KB-3-1 cells, and it was concluded that activity of these analogues was strongly correlated with DNA adduct formation.[153]

7. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As our model of cancer as a complex and molecularly heterogeneous disease sharpens, biomarker-driven drug selection for precision therapy will have an increasingly central role in cancer chemotherapy, both shaping the discovery and development of novel therapeutics, and giving rise to new companion diagnostic strategies for the more effective use of traditional drugs. Drugs that modify the structure of DNA and target cancer cells by interfering with DNA synthesis are current first line medications, but there remain drawbacks of resistance and side effects caused by poor selectivity toward tumor cells. In several studies carried out over the last 30 years, researchers have characterized the relationship between drug-induced DNA adducts and biological responses. These data suggest that the functional basis of inducing modifications in DNA may offer new opportunities for selecting susceptible patients on the basis of monitoring DNA adduct formation as a predictive diagnostic marker.

Much of the available data regarding the relationship between drug-induced DNA alkylation and biological responses are from *in vitro* studies aimed at understanding mechanisms of drug action or understanding biochemical factors that alter the susceptibility of cells toward these drugs, such as expression of GSH, DNA repair, transport, or bioactivation enzymes. In several recent studies, there has been further investigation about whether *in vivo* or patient responses can be predicted by DNA modification profiles. As reviewed herein, for a large majority of the preclinical studies of the relationship between drug action and adduct formation, there was a positive correlation observed between DNA adduct levels and response. These include analysis of samples such as from cell lines, tumor xenografts, or blood cells derived from cancer patients. In the case of clinical studies, a correlation between DNA adducts and response in samples such as xenografts, tumor biopsies, and blood cells derived from cancer patients was less reliable but could be established nonetheless most of the time. This discrepancy is not surprising since *in vivo* studies, particularly when the end point is patient responsiveness, are a more complex and potentially confounded situation, and therefore, prediction of drug efficacy is expected to be more difficult. These results are very encouraging, particularly considering that they come from a significant number of studies performed over a long period of time, on different samples, from different research groups, and analyzed with different analytical techniques. The high prediction of cytotoxicity from DNA adducts further emphasizes their biological importance in inducing cancer cell death and also emphasizes the value of understanding mechanisms of toxicity to identify candidate biomarkers. Lastly, DNA adducts benefit from their chemical stability and drug specificity.

There remain insufficiently addressed aspects and potential drawbacks regarding the application of DNA adduct monitoring in clinical settings. These issues include better definition of exposure strategies (i.e., *in vivo* therapeutic, subtherapeutic, and *ex-vivo* exposure). Monitoring DNA adducts in tumor biopsies or surrogate tissues after the first round of chemotherapy allows direct measurement of the susceptibility of a patient to a DNA modifying drug at therapeutic and therefore realistic concentrations. However, this approach involves the patient undergoing potentially lethal treatment with the disadvantage of experiencing unwanted side effects, not getting the best treatment, or developing resistance. Therapy-induced DNA adducts may be seen as a limitation for their use as biomarkers compared to conventional biomarkers that do not involve inducing any perturbation (e.g., KRAS mutations, HER2 overexpression, or hormonal receptor status).

As an alternative, DNA adduct monitoring after microdose administration of the DNA modifying drug has the advantage of the patient receiving a low dose of the drug that is too low for inducing side effects. A challenge of monitoring DNA adducts upon microdose exposure concerns the availability of analytical approaches suitably sensitive to detect drug-induced DNA adducts resulting from such low microdoses.

A third strategy is DNA adduct monitoring in isolated tumor samples or surrogate tissues treated *in vitro* with DNA alkylating drugs (*ex-vivo* exposure). This strategy poses the lowest risk for the patient since it does not involve any patient treatment to obtain sensitivity information. Additionally, this strategy could be envisioned to allow for the evaluation of several DNA alkylating drugs as candidate treatment options instead of only one drug. However, *in vitro* sensitivity testing bears greater uncertainty regarding whether DNA adducts are predictive of *in vivo* responses. As a general limitation, the use of DNA adducts as biomarkers requires first applying a chemical exposure and then measuring the biological response, rather than a direct predictive read out of a marker inherently present in the biological sample. This limitation, however, is directly related to a major advantage of DNA adducts, namely, their high biological relevance since they reflect the response of the system to a relevant perturbation, rather than its resting status.

Other drawbacks include uncertainty over the type of tissue to analyze and the timing of sample collection. In most studies, samples have been collected over the first few days after initiation of chemotherapy (or after diagnostic microdosing). However, it remains unclear which is the best sampling time for translation of this research into useful diagnostics tests. Furthermore, differences in rates and efficiencies of DNA repair could influence adduct levels. For example, Bosken et al.[166] and Wang et al.[83] reported that responses to platinum-based chemotherapy in NSCLC patients depended on DNA repair capacity (DRC) in cultured T lymphocytes. DRC was determined by measuring the repair of a benzopyrene-DNA adducted plasmid transfected into patient-derived lymphocytes as a surrogate for nucleotide excision repair of cisplatin-DNA adducts. There was a significant inverse correlation between DRC and patient survival. While a thorough review of how to account for DNA repair capacity is outside the scope of this perspective, these results emphasize the importance of DNA repair processes for determining patient outcomes and therefore the need for appropriate sample collection timing for clinical samples. These concerns would be best answered in head-to-head clinical comparisons, with separate studies for each cancer drug and cancer type.

Most of the *in vivo* studies have utilized surrogates for tumor tissue such as buccal cells, lymphocytes, and PBMCs owing to the poor availability of tumor biopsy tissue after initial diagnosis. There are ethical and patient risk concerns related to additional invasive tumor biopsy sampling that makes tumor

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data analysis of drug−DNA adduct formation and repair difficult from a clinical trial perspective. Until this approach is clinically validated in a large prospective clinical trial, the measurement of drug−DNA adducts as a companion diagnostics strategy to guide existing chemotherapy drug selection or for the development of new drugs remains premature.

A further difficulty centers on the extremely low analyte abundance and difficulties in the measurement of adducts, but in the past years, techniques for analysis of DNA adducts have evolved and improved enormously in sensitivity and specificity. Most classical methods, such as ^{32}P-postlabeling and immunoassays, lack the sensitivity, and specificity necessary when analyzing patient-derived samples, and others are not compatible with the high-throughput expected in clinical applications. The entrance of mass spectrometry to the field of DNA adduct analysis has been transformative and represents the current state-of-the-art in terms of specificity and sensitivity. The high sensitivity and precision of AMS for measuring carbon isotope ratios has uniquely enabled drug−DNA adducts in clinical samples for the development of predictive diagnostic tests, particularly for diagnostic microdosing. While AMS does not provide direct structural information and requires specialized equipment and expertise compared to typical LC-MS approaches, it can be practical for clinical use as central testing laboratories are commonly utilized for other specialized tests and has greatly advanced studies in the context of platinum-based therapeutics. Future research to address increasing the sensitivity and throughput of DNA adduct analysis technologies, as well as practical improvements in sample preparation, are expected in the next few years to have a significant impact on improving DNA adduct analysis and thereby allow the more general feasibility of measuring drug-induced DNA adducts in clinical settings.

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Notes

The authors declare no competing financial interest.

Biographies

Alessia Stornetta was born in Bellinzona, in the Italian-speaking region of Switzerland. She completed her B.S. degree (2010) and M.S. degree (2012) at the ETH Zurich, the Swiss Federal Institute of Technology in Zurich, Switzerland. She has carried out doctoral research at the University of Minnesota and the ETH Zurich. At the time of writing, Alessia is close to completing her dissertation research project, which focuses on the development of bioanalytical techniques for evaluating chemical-induced damage to DNA and their use for understanding factors that influence the susceptibility of cells to chemical toxicity.

Dr. Maike Zimmermann received her Ph.D. in Biochemistry in 2012 from the University of Bielefeld in Germany. She conducted her graduate research at the Department of Pharmacology, University of California (UC), Davis with a focus on cancer cell cycle regulation, DNA damage response, and estrogen receptor antagonism. In 2013, she joined the UC Davis Department of Internal Medicine and Accelerated Medical Diagnostics as a Postdoctoral Scholar to perform research on diagnostic microdosing in cell culture, animal models, and cancer patients. This work utilizes Accelerator Mass Spectrometry to predict patient response to several cytotoxic chemotherapy drugs.

Dr. George Cimino obtained his Ph.D. in Biochemistry from UC Irvine, where he used biophysical spectroscopy to study the repair of UV-induced damage in DNA. As a postdoc at UC Berkeley, he utilized psoralens to probe secondary and tertiary structures in macromolecular nucleic acid complexes. He continued work in the psoralen field with the application of psoralen-monoadducted oligonucleotides to diagnostics and then with the development and commercialization of DNA cross-linking agents for the inactivation of pathogens in transfusion products. In 2012, he joined Accelerated Medical Diagnostics to exploit the use of accelerated mass spectrometry with chemotherapy agents for predictive cancer diagnostics.

Dr. Paul Henderson received his Ph.D. in Organic Chemistry from the Georgia Institute of Technology in 1999, where he studied DNA-cleavage upon exposure to light. He then obtained a NIH Postdoctoral Fellowship to join John Essigmann at MIT, where he studied the mutagenic effects of DNA oxidation using a bacteriophage model. In 2002, he joined Lawrence Livermore National Laboratory, where he adapted the use of accelerator mass spectrometry to cancer research in collaboration with UC Davis. Since 2009, Paul has split his time between UC Davis and Accelerated Medical Diagnostics to validate the use of diagnostic microdosing in cancer therapy.

Dr. Shana J. Sturla was born in Brooklyn, NY. She obtained her B.S. degree in Chemistry at UC Berkeley in 1996 and Ph.D. degree in Chemistry at MIT in 2001. As a postdoctoral fellow of the American Cancer Society, she carried out research under the guidance of Stephen Hecht at the University of Minnesota concerning mechanisms of tobacco carcinogenesis. From 2004−2009, she was a Professor at the University of Minnesota, and since 2009 has been Professor of Toxicology at the ETH Zurich in Switzerland. Researchers in her lab address the chemical basis of disease incidence and treatment outcomes by investigating relationships among chemical structure, biotransformation, and cellular responses. Their website is www.toxicology.ethz.ch.

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ABBREVIATIONS

AMS, accelerator mass spectrometry; AAS, atomic absorption spectroscopy; AF, acylfulvene; AMS, accelerator mass spectrometry; AUC, area under the curve; BER, base excision repair; BRCA1/2, breast cancer genes 1 and 2; CHO, Chinese hamster ovary; CTC, circulating tumor cell; dGuo, deoxyguanosine; DMC, 10-decarbamoyl mitomycin C; DOX,

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Chem. Res. Toxicol. 2017, 30, 388−409

doxorubicin; DRC, DNA repair capacity; DSB, double strand break; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FACS, fluorescence-activated cell sorting; FA, Fanconi anemia; FDA, fluorescein diacetate assay; GDF, growth delay factor; GFR, glomerular filtration rate; GSH, glutathione; HAPs, hypoxia-activated prodrugs; HbA1c, hemoglobin A1c; HCR, host-cell reactivation; HCT, hematopoietic cell transplantation; HER2, human epidermal growth factor receptor 2; HPLC, high performance liquid chromatography; HPV, human papilloma virus; ICC, immunocytochemistry; ICL, interstrand cross-link; ICP, inductively coupled plasma; IHC, immunohistochemistry; INR, international normalized ratio; KRAS, Kirsten rat sarcoma viral oncogene homologue; LC, liquid chromatography; LOQ, limit of quantification; LSC, liquid scintillation counting; LSI, laser spray ionization; MC, mitomycin C; MS, mass spectrometry; MTA, microculture tetrazolium assay; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; MTT, 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; NER, nucleotide excision repair; NSCLC, non-small-cell lung cancer; PBLs, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cell; PDX, patient derived xenograft; PM, porfiromycin; PSA, prostate specific antigen; PTGR1, prostaglandin reductase 1; QPCR, quantitative polymerase chain reaction; RAGE, rotating field gel electrophoresis; RT, radiation therapy; SF, sulforaphane; SRB, sulforhodamine B; SSB, single strand breaks; T/C, treated/control; TPZ, tirapazamine; VEGFR, vascular endothelial growth factor receptor; WBC, white blood cell; γH2AX, gamma phosphorylation of histone H2AX

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