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DNA as a target for anticancer compounds: methods to determine the mode of binding and the mechanism of action

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Small molecules that bind to DNA are extremely useful as biochemical tools for the visualization of DNA both *in vitro* and inside the cell. Additionally, the clinical significance of DNA-binding compounds can hardly be overstated, as many anticancer regimens include a compound that binds to and/or modifies DNA. Although many of the important DNA-binding anticancer drugs were discovered in phenotypic, cell-based screens, *in vitro* experiments have been developed that enable a precise determination of how a compound interacts with DNA. This review provides a summary of the assays that should be performed when it is suspected that DNA may be a target for a given small molecule. A battery of *in vitro* assays readily distinguishes between DNA intercalation, DNA groove binding, and the inhibition of topoisomerases. Further cell-based investigations can implicate a direct effect of a compound on DNA within the cell. Together, these assays are powerful tools to determine the mechanism of previously discovered molecules, and will be crucial to the discovery of the next generation of DNA-binding anticancer drugs.

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Cancer is now the number one killer disease in the United States. Despite the recent excitement about personalized anticancer therapies, such drugs currently represent a small fraction of anticancer agents. A large percentage of chemotherapeutic anticancer drugs are compounds that interact with DNA directly or prevent the proper relaxation of DNA (through the inhibition of topoisomerases). In addition, DNA-targeting cancer drugs continue to be developed, as evidenced by the recent approval of belotecan [1]. Given the applications of DNA-targeting drugs for cancer and beyond, the further discovery and characterization of such compounds are of considerable interest. This review outlines diagnostic experiments

useful in characterizing noncovalent drug–DNA interactions *in vitro* and inside the cell.

Intercalating agents

Drug–DNA interactions can be classified into two major categories, intercalation and groove binding (Figure 1). Intercalation involves the insertion of a planar molecule between DNA base pairs, which results in a decrease in the DNA helical twist and lengthening of the DNA [2]. Although there is a significant free energy cost for the establishment of the intercalation cavity (approximately 4 kcal mol⁻¹), favorable contributions (hydrophobic, ionic, hydrogen bonding, and van der Waals) result in association constants of 10⁵ to 10¹¹ M⁻¹ [3]. Although intercalation has been traditionally associated with molecules containing fused bi/tricyclic ring structures, atypical intercalators with nonfused rings systems (Figure 2) may be more prevalent than previously recognized [4•]. Although DNA intercalators have been used extensively as antitumor, antineoplastic, antimalarial, antibiotic, and antifungal agents, not all intercalators are genotoxic (defined by the ability to alter a cell's genetic material as a means of inducing a toxic effect). The presence of basic, cationic, or electrophilic functional groups is often necessary for genotoxicity [4•,5].

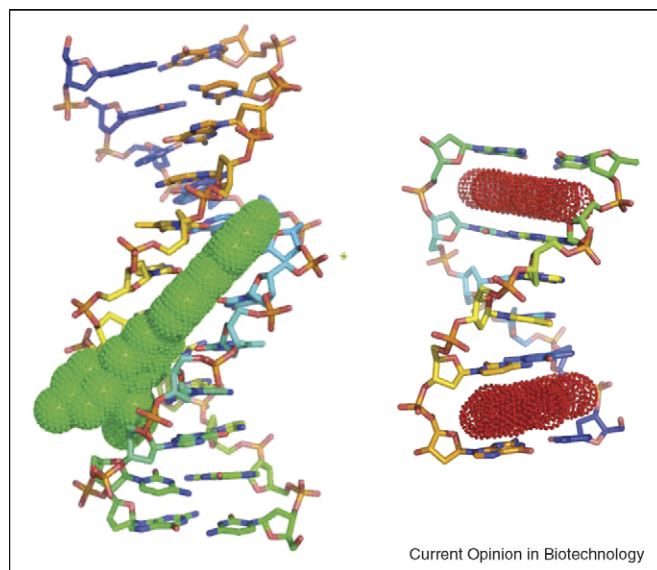
Groove-binding compounds

Groove binding, unlike intercalation, does not induce large conformational changes in DNA and may be considered similar to standard lock-and-key models for ligand–macromolecular binding [3]. Groove binders are usually crescent-shaped molecules that bind to the minor groove of DNA (Figure 2). They are stabilized by intermolecular interactions and typically have larger association constants than intercalators (approximately 10¹¹ M⁻¹), since a cost in free energy is not required for creation of the binding site [3]. Like intercalators, groove binders also have proven clinical utility as anticancer and antibacterial agents, as exemplified by mitomycin (which is also a DNA crosslinker) [6]. Notably, the anthracyclines, a class of clinically important compounds with antineoplastic and antibacterial properties, take advantage of both modes of binding as they possess an intercalative unit as well as a groove-binding side chain.

Experimental considerations – DNA binding

If a newly discovered compound is suspected of targeting cellular DNA, a battery of simple *in vitro* experiments can be performed to readily determine whether the compound physically interacts with DNA. For example, if in the presence of DNA a red-shift is observed in a

Figure 1



Groove binding of Hoescht 33258 to the minor groove of DNA (left, NDB structure 8bna) [49] and the intercalation of ellipticine into DNA (right, NDB structure 1z3f) [50].

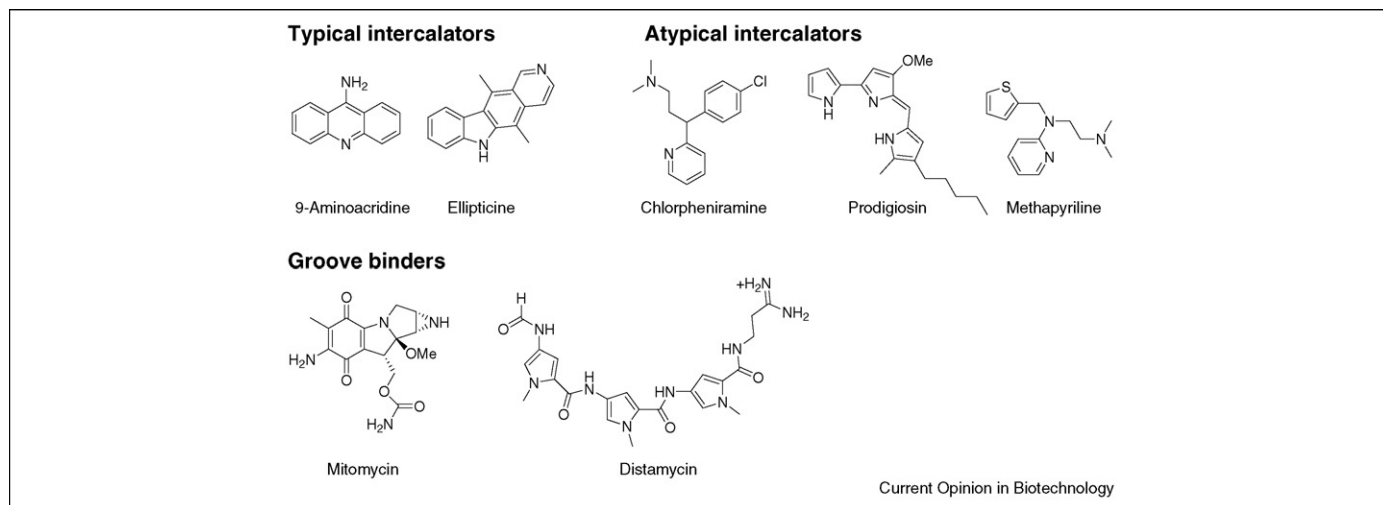
compound's UV-vis absorption, this may suggest an interaction with DNA [7]. More conclusively, DNA thermal denaturation studies can be performed. In these experiments duplex DNA is thermally denatured into single-strand components in the presence and absence of compounds. Compounds that physically associate with DNA typically stabilize the duplex, hence increasing the midpoint denaturation temperature (T_m) (Figure 3a) [8].

Fluorescent compounds may have increased fluorescence emissions when bound to DNA [9]. For example, the

fluorescence of the intercalator ethidium bromide (EtBr) increases 24-fold in the presence of DNA, while Hoescht 33258, a groove-binding molecule, increases by 140-fold upon DNA binding [7]. A compound's fluorescence excitation wavelength may also shift upon interaction with DNA [7]. Furthermore, changes in the circular dichroism spectrum of DNA in the presence and absence of varying ratios of drug:DNA can also indicate an interaction with DNA [10,11].

Isothermal titration calorimetry (ITC) is a sensitive technique in which compound is titrated into a solution of

Figure 2



Structures of typical intercalators, atypical intercalators, and groove binders.

(length³), viscosity measurements are very sensitive to changes in the length of DNA; the application of viscosity measurements is limited only by the compound's aqueous solubility. If a circular plasmid is used, the viscosity peaks and then declines with increasing concentrations of the compound, indicative of the DNA changing from a negative-supercoiled to a relaxed and finally a positive-supercoiled state (Figure 3b) [13].

Since EtBr intercalates DNA through interactions with the minor groove, the displacement of EtBr (quantified by fluorescence) by the titration of a compound is suggestive of an intercalative or minor groove binding [14,15]. EtBr is particularly suitable as a reporter because of its 24-fold decrease in fluorescence (as well as shifts in its excitation spectrum) when displaced from an intercalation site [7].

Fluorescence contact energy transfer experiments involve the monitoring of the emission spectrum of a fluorescent compound and obtaining the excitation spectrum in the presence of DNA [7]. The appearance of an excitation band absorption wavelength of DNA (260 nm) indicates a close interaction and transfer of energy between the DNA base pairs and the small molecule. Although originally thought to be exclusive to intercalation, certain groove-binding compounds are capable of fluorescence contact energy transfer, therefore other experiments are required to corroborate the mode of binding [16].

Although viscosity can conclusively identify intercalators, electric linear dichroism can simultaneously distinguish between intercalative and groove-binding modes of interaction [17]. Pulses of electric current align linear DNA strands such that they are parallel to the electric field vector (Figure 3c). The absorbance of plane polarized light parallel ($A_{||}$) or perpendicular (A_{\perp}) to the field is quantified in the presence/absence of compound and can be used to determine the orientation of the small molecule with respect to DNA. If the absorbance of plane-polarized light parallel to the electric field is less than the polarized light perpendicular to the field (negative dichroism), this is suggestive of an intercalative mode of binding. Conversely, a positive dichroism is indicative of a groove binder. This method has the advantage of requiring a low drug:DNA ratio (1:10) and is sensitive to anisotropic interactions. Electric linear dichroism has been used to successfully identify sequence-dependent interactions of various clinically used DNA-binding compounds [17]. More impressively, it has been used to identify specific GC rich sequences that allow the groove binders Hoescht 33258, DAPI, and berenil to assume a nonclassical intercalative mode of binding [17,18].

Topoisomerase assays

Topoisomerase-based gel assays have also been widely used to evaluate compounds for their ability to intercalate

into DNA. Topoisomerases are enzymes that control DNA winding and eliminate knots and tangles from DNA. These enzymes have essential roles in replication and transcription, and allow for correct chromosomal configuration and partition. There are two principal types of topoisomerases, I and II, which cause single-stranded and double-stranded breaks in supercoiled DNA, respectively. Of the human type I enzymes — Topo I, Topo III α and Topo III β — only Topo I has been confirmed as a target of anticancer drugs (camptothecin-based derivatives: irinotecan, topotecan, and belotecan) [19,20]. Of the type II enzymes, Topo II α and Topo II β are encoded by different genes and possess discrete expression levels. Topo II α 's expression is regulated in a proliferation-dependent manner where rapidly dividing cells and tissues have pronounced levels [21,22]. The intercalating anthracyclines (doxorubicin and daunorubicin) and etoposide (Etop) are examples of compounds in clinical use that exert their anticancer activity by reducing the activity of Topo II α [23]. Conversely, growth does not influence the expression of Topo II β ; this enzyme is present in all cells and is expendable for cell viability (except for *in vivo* neural development), thus probably making it a poor anticancer target [24,25].

Topoisomerase assays: intercalation or inhibition?

Evaluation of topoisomerase activity is typically performed through gel electrophoresis assays. In these experiments, negatively supercoiled (Experiment 1, Figure 3d) or relaxed (Experiment 2, Figure 3d) plasmid DNA is treated with Topo I in the presence of increasing concentrations of the compound being investigated. By performing Experiment 1 (with supercoiled DNA) first, a distinction can be made between compounds that have no activity and those that are either inhibitors or intercalators. To subsequently differentiate topoisomerase inhibitors from DNA intercalators, Experiment 2 needs to be performed. Compounds that are Topo I inhibitors will prevent Topo I from changing the state of the relaxed DNA, whereas in the presence of an intercalator Topo I will convert the relaxed DNA into a supercoiled state (see Experiment 2, Figure 3d). An example of Experiment 2 (a Topo I gel assay with a DNA intercalator) is shown in Figure 3e. In this experiment, relaxed DNA was treated with increasing concentrations of compound MLN944 in the presence of Topo I. As indicated by the gel, the DNA is converted from a relaxed to a supercoiled state, indicating that MLN944 is a DNA intercalator.

Compounds that reduce topoisomerase activity: noncovalent inhibition or poison?

Although the topoisomerase gel electrophoresis assay has been commonly used to identify intercalators, the distinction between catalytic and poisoning modes of topoisomerase inhibition is not often made. Catalytic inhibitors may prevent the binding of DNA to topoisomerase,

stabilize noncovalent DNA–topoisomerase complexes, or prevent the binding of ATP to the enzyme [26^{••}]. Topoisomerase poisons, however, stabilize a covalent DNA–topoisomerase complex, thus disabling the enzyme [26]. Webb and Ebeler have developed assay conditions that allow the distinction between catalytic inhibition and poisoning [27]. The observation of a dose-dependent increase of the ‘nicked’ form of the plasmid, resulting from the cleavage of a phosphodiester bond of one of the DNA strands, indicates that the compound is a topoisomerase I poison. The use of a known nonintercalating, nonpoisoning topoisomerase I inhibitor (morin), a topoisomerase I poison (camptothecin), and nonpoisoning intercalator (EtBr) as reference compounds can be helpful in preventing the misinterpretation of results obtained from the assay [27].

Once a stable DNA–topo complex forms inside the cell, the damaged DNA is recognized, initiating p53-dependent cell death [28]. Although a variety of intercalators and groove binders have been documented as topoisomerase I or II targeting agents, the therapeutic properties of compounds that are dual topoisomerase I and II inhibitors are currently the subject of much interest [29,30].

Sequence specificity

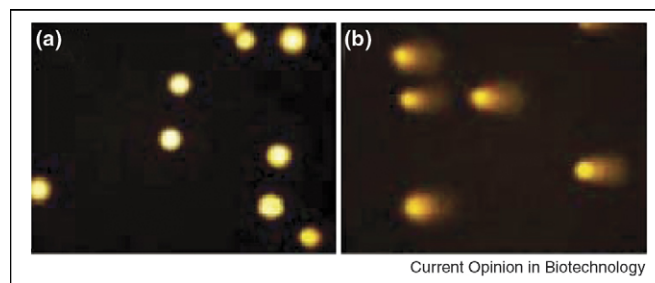
Once a mode of DNA binding has been identified for a compound, it may be necessary to determine its DNA sequence specificity. Although electric linear dichroism has been used successfully to identify sequence selective binding [17], the most commonly used technique is the DNase footprinting assay. In this assay a 3′-³²P labeled DNA fragment of known sequence composition is incubated with DNase in the presence of increasing concentrations of compound [31]. The resulting differential cleavage pattern indicates that bases were protected from enzymatic digestion, and thus, the precise bases the compound binds. This method allows for the investigation of many sequences through the use of reasonably large DNA fragments.

The use of restriction endonuclease protection selection and amplification (REPSA) allows the combinatorial assessment of a large library of DNA sequences under relevant mild physiological conditions [32]. The sequence specific interaction of a molecule with DNA inhibits DNA cleavage by a restriction endonuclease, and upon multiple amplifications and selections, the consensus sequence is identified [33^{••}].

Evaluation of DNA binding in the cell

Although the experiments described thus far indicate whether a small molecule binds to DNA *in vitro*, methods to validate compound binding to DNA inside the cell are limited. Fluorescence microscopy may be used to demonstrate a molecule’s ability to penetrate the nuclear mem-

Figure 4



The increase in the comet tail length in H460 cells after treatment with the DNA crosslinker oxaliplatin. (a) Control cells. (b) Oxaliplatin-treated cells. Figure reprinted from manuscript of Almeida *et al.* [40].

brane and preferentially localize to the nucleus [34,35]. Such experiments can put a drug at the ‘scene of the crime’ but do not conclusively demonstrate that the compound binds DNA in the cell.

The comet assay allows the assessment of low levels of DNA damage in single cells treated with compound [36[•]]. Following compound treatment, cells are embedded on a thin agarose gel. Removal of cellular proteins by lysis, followed by DNA unwinding (under alkaline or neutral conditions) and electrophoresis allows damaged/broken DNA fragments to migrate away from the nucleus. Following poststaining, the fluorescence of the head and tail, and the tail length/moment, can be quantified. The tail length/moment is a measure of the level of DNA damage present (Figure 4) [37^{••}]. Neutral DNA unwinding conditions allow for the assessment of double-stranded DNA breaks, while alkaline conditions permit the detection of single-stranded breaks, alkaline labile sites, oxidative damage, and DNA–DNA or DNA–protein crosslinking [36[•],38]. Double-stranded DNA breaks have been shown to correlate with the level of apoptosis in cells induced by hydrogen peroxide, gamma radiation, and DNA-targeting chemotherapeutics such as bleomycin [39]. The comet assay also allows the investigation of DNA repair kinetics at the level of single cells [40].

Case study

Prodigiosin (Figure 2), a natural red pigment produced by *Streptomyces* and *S. marcescens*, has demonstrated immunosuppressive and potent anticancer properties [41–43]. Although the planar, unfused tripyrrolic molecule has been shown to first, uncouple vacuolar H⁺-ATPase by promoting H⁺/Cl[−] symport [43] and second, regulate mitogen-activated protein kinases, other modes of action may contribute to its biological activity [44]. Based on the observed binding of prodigiosin to DNA by absorption and fluorescence spectroscopy [45,46], Montaner *et al.* investigated the possibility of DNA and topoisomerases as a third possible target [29]. Prodigiosin is fluorescent and preferentially localized to the nucleus

by microscopy [29]. *In vitro* Topo I gel assays demonstrated that prodigiosin intercalates DNA [29]. Through the use of prerelaxed plasmid DNA, increasing concentrations of prodigiosin prevented supercoiling by both Topo I and II, identifying prodigiosin as a dual Topo I and II inhibitor [29]. Cell-based immunoblotting for topoisomerase by the link assay demonstrated the presence of stable Topo I–DNA and Topo II–DNA complexes in the presence of compound and validated topoisomerases as cellular targets [29]. Agarose gel electrophoresis assays demonstrated prodigiosin's ability to increase the oxidative damage of supercoiled plasmid DNA under slightly acidic conditions (pH 6.8) compared to weakly basic conditions (pH 7.4) [29]. As the extra cellular pH in solid tumors is typically between pH 6.6 and 7.0 versus pH 7.1–7.6 in normal tissue [47], this may allow for selective toxicity. Furthermore, in the presence of Cu²⁺, prodigiosin-mediated oxidative damage to supercoiled plasmid DNA increased markedly, as visualized by agarose gel electrophoresis [29,46]. The higher concentrations of Cu²⁺ in breast cancer tissue than in noncancerous breast tissue may further enhance the compound's ability to effectively target breast cancer tumors [29]. In summary, while multiple mechanisms have been proposed to explain prodigiosin's antitumor activity, DNA damage mechanisms involving dual Topo I/II inhibition and Cu²⁺ or pH-dependent oxidative damage may contribute to the promising antitumor properties of prodigiosin [48].

Outlook

Although the next generation of oncology therapeutics will likely focus on 'targeted' small molecule therapies and protein/antibody-based drugs, DNA-binding compounds have proven their utility and will continue to be a staple of anticancer regimes. Thus, the discovery of new DNA-targeting drugs that have improved toxicity and pharmacokinetic profiles is of great importance. Through experiments outlined herein, it should be straightforward to rapidly determine if and how a novel cytotoxin interacts with DNA.

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