Newer Approaches to the Study of the Mechanisms of Action of Antitumor Antibiotics

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Nature displays extraordinary ingenuity in the design and biosynthesis of antibiotics (Figure 1). While antibiotics have attracted a great deal of interest,[1,2] those for which one may formulate a mechanism of action in chemical terms with any degree of confidence are few in number.[2] The particular area I wish to address in this Account concerns the mechanism of action of antitumor antibiotics. Although determining the mechanism of antibiotic action is a chemical problem, it clearly requires a multidisciplinary approach.

Considerable pharmacological evidence both in vivo and in vitro, including the inactivation of viruses and antimitotic, mutagenic, and cytological effects, indicates that DNA is a critical cell target for many antitumor antibiotics owing to the sensitivity of its cellular function to chemical alteration.[2] Such drugs may inhibit the cellular functions of nucleic acids at the level of nucleotide metabolism, by direct inhibition of replicative enzymes or by interactions of various kinds with nucleic acid templates.[2] DNA is an attractive drug target for study since it has, more or less, a defined geometry.[2,3]

The types of specific information one requires when investigating an antibiotic inhibitor of nucleic acid functions fall into three categories:

1. The Antibiotic.

(i) Does the drug react with a replicative enzyme or protein, DNA polymerase, RNA polymerase, gyrase, topoisomerase, helix destabilizing protein or directly with the nucleic acid template?

(ii) Does the drug require specific activation, reduction, oxidation, or protonation, and if so where?

(iii) Does it require a cofactor such as metal ions or O_2?

(iv) What are its conformation, absolute configuration, and dimensions in relation to the DNA receptor; i.e., are there specific requirements for molecular recognition?

2. DNA—The Antibiotic Target.

(i) Is a particular conformation or type of DNA A, B, D, β kinked or Z[3] uniquely responsive to attack by the antibiotic?

(ii) Which topological form of DNA is especially sensitive to the antibiotic—helix, random coil, linear, circular, supercoiled?[3]

(iii) Is a particular base sequence in the DNA recognized by the antibiotic?

(iv) How is the action of the antibiotic influenced by proteins such as histones associated with the DNA template?[3]

3. Types of Chemical Interaction between Antibiotics and DNA.

(i) Which of the following specific types of chemical modification (or which combinations) does the antibiotic cause on the DNA: (a) covalent binding to bases or phosphates; (b) formation of apurinic or apyrimidinic sites; (c) interstrand cross-linking or DNA–protein links; (d) single strand breaks; (e) double strand breaks; (f) intercalation or bis intercalation; (g) nonintercalative and noncovalent binding?

(ii) Does the antibiotic show any DNA groove preference?

(iii) What are the chemical mechanisms of the particular lesions produced by a given antibiotic?

(iv) Which of these chemical lesions (if any) is related to the primary cytotoxic event and which (if any) may be related to specific toxic effects?

Methods for Studying Antibiotic Action

Some of the above questions, especially those dealing with static aspects, yield to conventional methods. For example, high-field NMR is especially informative with regard to oligonucleotide configuration and conformation,[3a] sites of antibiotic protonation,[4] and interaction of antibiotics with DNA[5] and with cofactors such as metal ions.[6] Electron microscopy may be used for the mapping of small molecules bound to nucleic acids.[7] X-ray diffraction is yielding discrete information on intercalative interactions in some drugs.[3b] Methods such as agarose gel electrophoresis[8] and alkaline elution techniques[9] are useful for discriminating between single and double strand breaks. The more dynamic aspects dealing with the chemical interaction of antibiotics with nucleic acids require different methods. The familiar chemical approach of simplifying the system by examining the reaction of the antibiotic with a nucleoside is often inappropriate and may be misleading since many antibiotics do not react with them and perforce require

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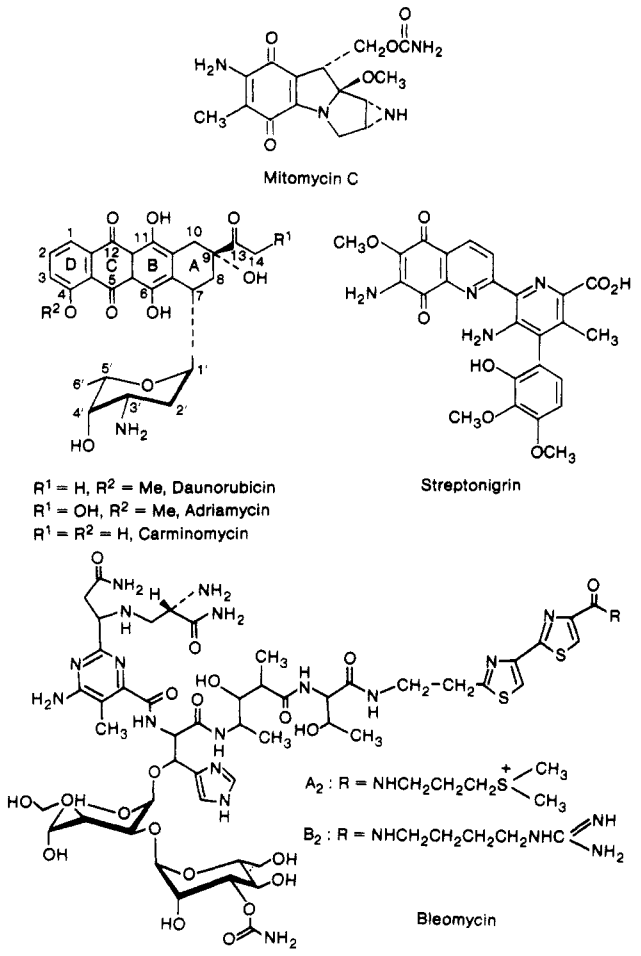

Figure 1. Structures of antibiotics mitomycin C, anthracyclines, steptonigrin, and bleomycin.

the full structure of duplex DNA to express their functions.

Clearly, one needs methods with which one may study the reactions of antibiotics with native DNAs directly and which are sufficiently sensitive to detect interactions characteristic of different conformations and topologies. Some of the most useful methods are those that employ enzymes that recognize and respond to a particular lesion, thereby giving unambiguous mechanistic information, and that are capable of detecting and distinguishing more than one simultaneous chemical lesion.

Ethidium Binding Assays

Particularly sensitive and adaptable methods based on the fluorescence characteristics of some intercalative dyes that may be used in conjunction with certain enzymes involved in cellular protection, growth, and repair are useful in this regard. A planar chromophore having dimensions compatible with the Watson–Crick duplex inserts between adjacent base pairs and is held there by van der Waals, stacking, and electrostatic forces. Ethidium bromide (Figure 2) binds intercalatively to duplex DNA with no apparent sequence selectivity (binding constant $K_b \approx 10^5$–10^7 M^{-1}) and in the process shows an enhancement in its fluorescence intensity of ca. 25-fold[10] as a result of reduced proton exchange leading to a longer lifetime for the excited state.[11] This property may be used to detect duplex DNA very sensitively (a practical lower limit of $<10^{-7}$ g of DNA has been estimated[12]). Assays have been devised using this principle[13–16] that can provide information on each of the types of chemical interaction listed above.

Interstrand cross-linking may readily be detected as follows (Figure 3). In a control experiment with linear DNA, the DNA will denature upon heating at 96 °C for 3 min followed by rapid cooling. Provided the pH is high (~11.8) this prevents the formation of short regions of self-complementarity so that potential ethidium intercalation sites are destroyed and the fluorescence falls to zero. In contrast, if a drug has introduced an interstrand cross-link, this will serve as the nucleation site for rapid denaturation[17] following the heating and cooling cycle. The concomitant observation of ethidium fluorescence corresponding to the proportion of the DNA molecules present that contain at least one cross-link therefore permits quantification of this DNA lesion[18,19] (Figure 2). This assay may be extended by employing DNAs of different base composition to examine the base dependency of cross-link formation.[18,19]

A second assay using supercoiled covalently closed circular (CCC) PM2-phage DNA may be used to detect and quantify single strand scission.[20,21] Supercoiled DNAs that occur naturally (Figures 3 and 4) are especially useful in exploring the effects of DNA topology on antibiotic reactivity and specificity.

The sensitivity of the assay may be increased by first relaxing the supercoils of the DNA with a topoisomerase enzyme (Figure 3)[13,18] whose function in biological systems appears to be to remove topological strain resulting from supercoiling ahead of the replicating fork.[15] When PM2-CCC-DNA is used, the sensitivity of the assay is such that one chemical event may be detected in a DNA of the equivalent of 6×10^7 daltons.[13]

The same sensitivity is obtained with an equivalent assay for detecting alkylation of DNA[22] (Figure 4).

Covalent Binding of Antibiotics to DNA

The mitosane antitumor antibiotics, of which mitomycin C (Figure 1) has received the most study, inhibit DNA synthesis. The cellular mechanism, as proposed by Iyer and Szybalski,[23] involves quinone reduction (plausibly by two successive 1-electron reductions since the presence of the obligatory semiquinone intermediate may be detected by EPR in vivo[24] and in vitro[25]) with

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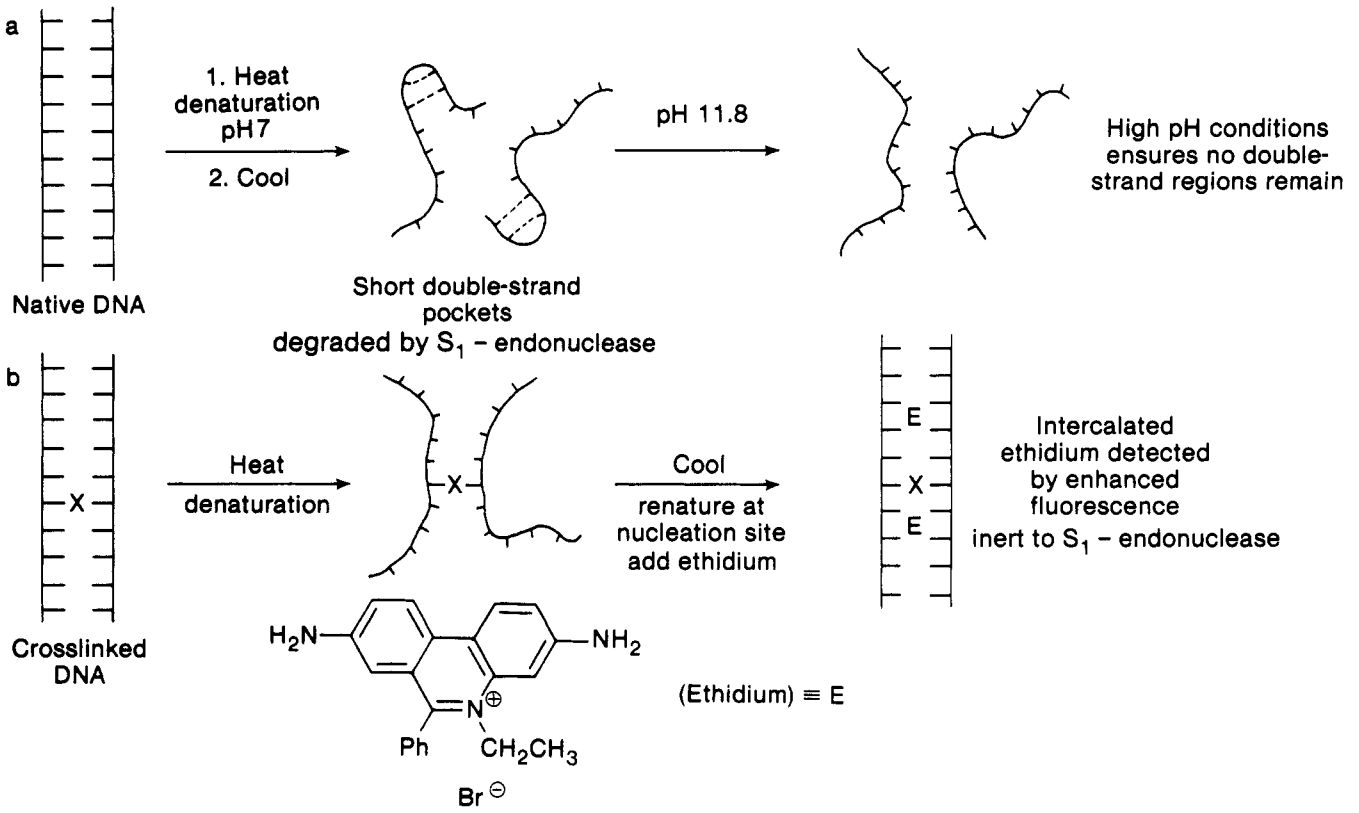

Figure 2. Ethidium binding assay and experimental conditions for determining DNA interstrand cross-linking.

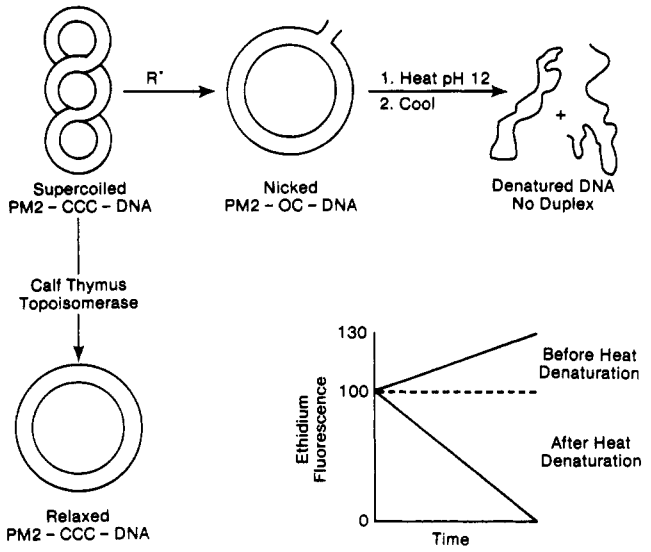

Figure 3. Ethidium binding assay for detecting single strand scission of supercoiled or topisomerase-relaxed covalently closed circular (CCC) DNA.

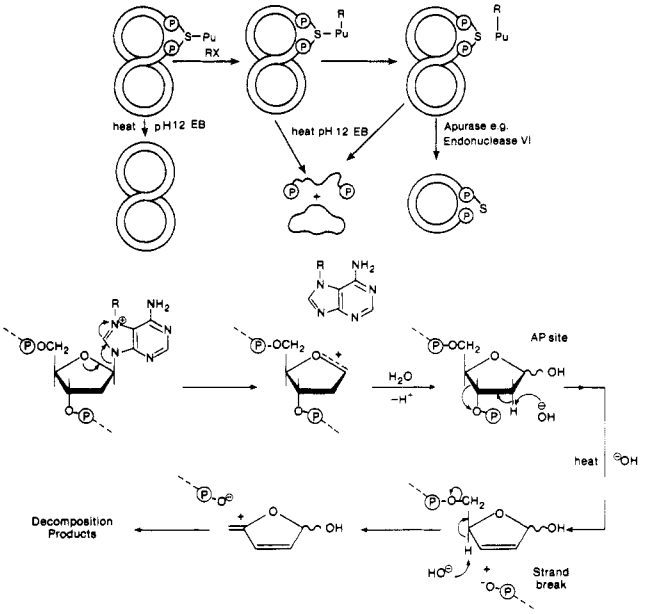

Figure 4. Reactions of DNA involved in the spontaneous loss of alkylated purine with generation of an AP site and subsequent cleavage by apurase or hydrolysis to form a DNA strand break.

concomitant loss of CH_3OH. The resulting transient-activated antibiotic is considered to bind covalently to DNA by both alkylation and formation of interstrand cross-links.[23] Mitomycin C reduced in situ binds to DNA and sterically impedes the intercalation of ethidium, causing a quantitatively proportionate decrease in enhanced fluorescence.[18] It can then be shown by employing DNAs of different base composition that activated mitomycin C shows a preference for reaction at (G + C) rich sites both in alkylation and in the concomitant interstrand cross-linking.[18]

DNA treated with agents such as $(CH_3)_2SO_4$, which are known to react preferentially at N-7 of guanine, exhibit markedly different time-dependent behavior from mitomycin C treated DNA when maintained at 37 °C and pH 11.8[26] (Figure 5).

At pH 11.8 the fluorescence intensity shows a slow recovery, so that with low levels of methylation (~4% deoxyguanosine residues modified as determined by radiolabeling techniques) one observes complete return of fluorescence. These phenomena are attributed to the conversion of 7-methyldeoxyguanosine to the zwitterionic form and partial denaturation of the DNA duplex

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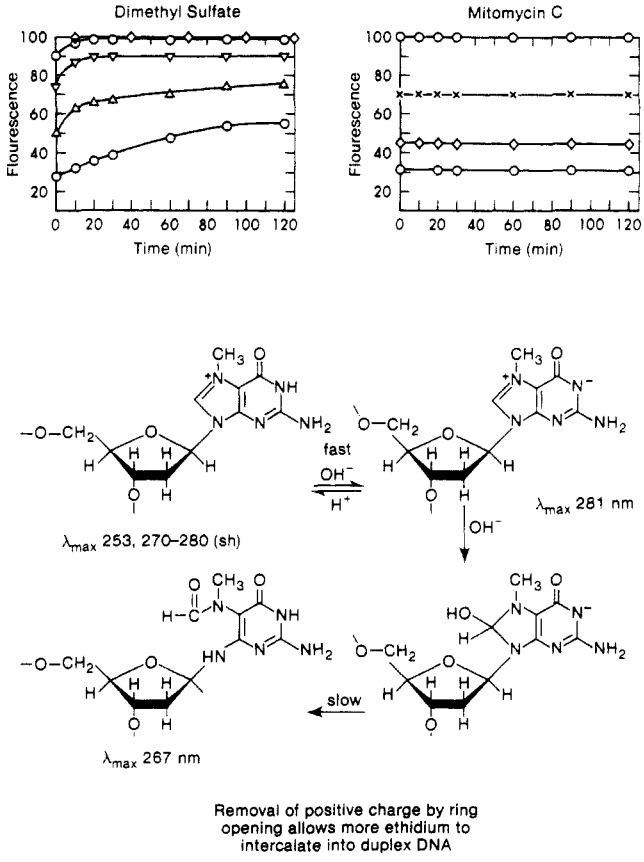

Figure 5. Ethidium binding assay for distinguishing 7-guanosine alkylation from reaction at other base sites.

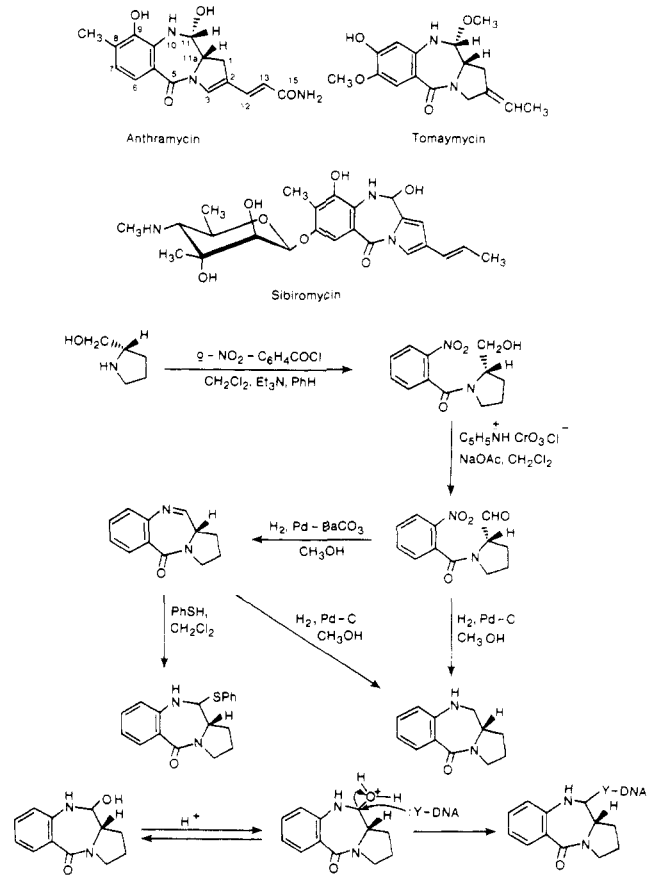

Figure 6. Structures of pyrrolo[1,4]benzodiazepinone antibiotics, synthesis of parent three-ring system, and possible mechanism of acid-promoted DNA binding.

with loss of ethidium binding sites. Base-catalyzed imidazole ring opening and the removal of the positive charge permit reannealing with concomitant return of ethidium intercalation sites with the attendent rise in fluorescence. This conclusion was substantiated by enzymatic hydrolysis of ^{14}C-labeled methylated DNA and identification of the two types of deoxyguanosine residues formed under the different conditions of the ethidium assay.[26]

In contrast, the mitomycin C–DNA complex shows no similar time dependence of the ethidium fluorescence values, from which it may be inferred that the antibiotic is bound at some other site on guanine that does not produce a charged species, a conclusion that has been reached independently involving a tritium exchange procedure.[27]

The possible sites of reactivity in the mitosanes include the aziridine, the carbamate, and the 7-NH_2 group.[23] Mitomycin B, upon reduction with $NaBH_4$ in situ, cross-links DNA.[28] Mitomycin B (which bears a 7-OCH_3 group in place of NH_2) in which the aziridine ring was opened with mild acid treatment was found to bind covalently to DNA after in situ reduction with $NaBH_4$ but not to cause interstrand cross-links.[28] It may be inferred, then, that the two reactive sites in the mitosanes that are involved in cross-linking (Figure 2) are the aziridine and probably the 10-carbamate moiety.

The Pyrrolo[1,4]benzodiazepinone family of antibiotics includes anthramycin, tomaymycin, sibiromycin (Figure 6), and neothramycins A and B, which inhibit macromolecular synthesis, by direct interaction with DNA templates.[29,30] Inspection of the structure and analogy with other antibiotics leads one to anticipate the 10,11-carbinolamine moiety to be the site of reactivity towards DNA. This expectation has been elegantly confirmed by Hurley and his co-workers.[30] The three-ring pyrrolo[1,4]benzodiazepinone system common to these antibiotics was synthesized (Figure 6) and found to bind to DNA like the parent anthramycin but to a lesser extent.[31] That the guanine base and minor-groove preference and the acid-catalyzed formation and reversal of the binding of this fragment[31] exactly parallel the behavior of anthramycin is in accord with the suggested aminal linkage formed by the reaction of the 2-NH_2 group of guanine in the minor groove.[30] (cf. Figure 7).

The saframycin antibiotics isolated from *Streptomyces laevendulae*, which exhibit promising antileukemic properties,[32] offer an interesting contrast with the pyrrolo[1,4]benzodiazepinone group. Saframycin A (Figure 7), which, like anthramycin, inhibits nucleic acid functions by direct interaction with DNA templates,[32] exhibits three different reactivities toward DNA. At lower pHs (6.5–5.0) it protonates preferentially on N–CH_3 (shown by 400 MHz ^1H NMR)[5] and displays rapid equilibrium binding to DNA.[33] In addition, saframycin A undergoes a slower acid-catalyzed rever-

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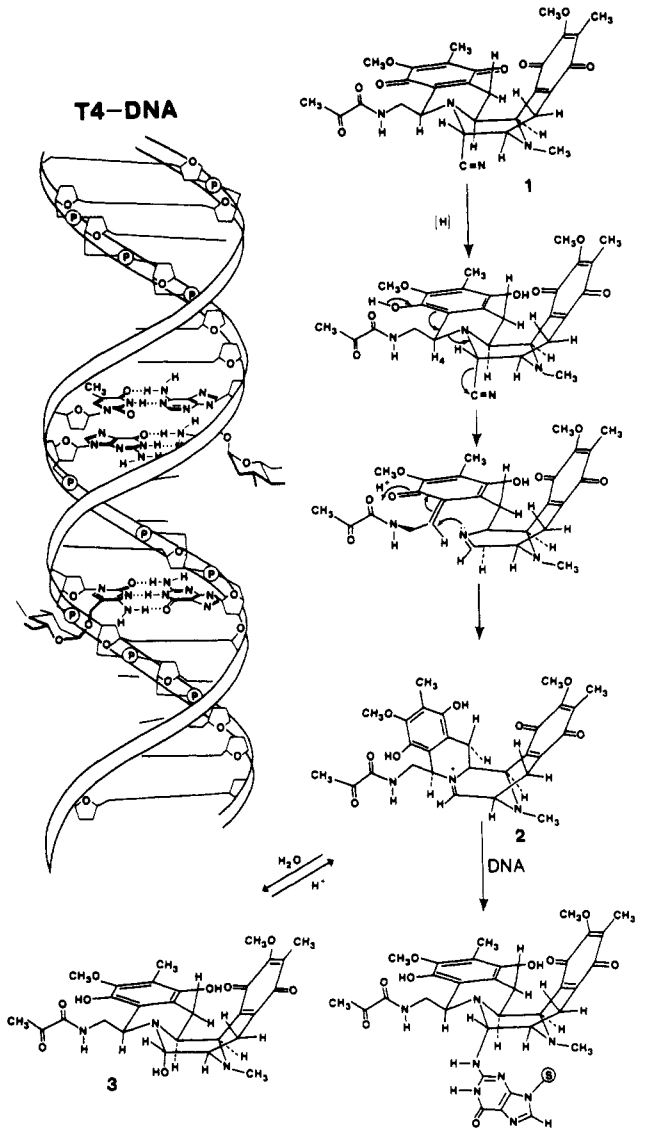

Figure 7. Enzymatic reduction of saframycin A (1) quinone moiety with concomitant loss of CN^-, ring closure to cyclic immonion ion (2), equilibration to saframycin S (3), or DNA minor-groove binding via an aminal linkage to guanine 2-NH_2. Depiction of T4 phage DNA in which major groove is occluded by α-glucosylated cytosine residues.

sible binding to (G + C)-rich sites. The fact that saframycin A, like anthramycin, binds to T4 DNA, in which the major groove is occluded because all the cytosine residues are α-glycosylated (Figure 7), indicates a preference for the minor-groove binding of this antibiotic.[33] The unprotonated, and therefore free, N-4 lone pair assists in loss of the nitrile leaving group, generating a reactive electrophilic immonium ion, which is capable of alkylating DNA at the 2-NH_2 of guanine in the minor groove. Independent evidence in accord with this finding on the site of binding has been obtained by ^{13}C NMR spectroscopy.[34]

The saframycins require a reducing cofactor for the expression of biological properties in vivo.[35] The increase in antibiotic binding by saframycin A in the presence of NADH is evident from the proportionate suppression of ethidium binding.[33] At the same time, the slow acid reversal of this (covalent) binding distinguishes it from equilibrium binding. Simultaneously, however, one observes single strand scission (but no double strand breaks) of circular duplex DNA by agarose gel electrophoresis.[33] Moreover, one may establish the chemical mechanism of this process as described below.

DNA Interstrand Cross-Linking

Although alkylation of DNA is more common by a factor of ~10 to 20, interstrand cross-linking is much more likely to be a lethal cytotoxic event because of the difficulty of repair.[10] Cross-linking may be detected by hyperchromicity or sucrose gradient sedimentation, but the ethidium binding assay (Figure 2) offers the advantage that one may often determine its sequential nature, follow its kinetics, and quantify it.

If λ-phage DNA (a linear DNA) is treated at 37 °C with mitomycin C reduced in situ with $NaBH_4$, the mixture after a time quenched at 0 °C and dialyzed to remove unreacted antibiotic and the purified antibiotic–DNA complex examined at 37 °C, a new phenomenon is observed.[18] The ethidium binding after a heat denaturation (96 °C/4 min) and cooling (22 °C) cycle increases with time, indicating the formation of a cross-link by a second chemical bond to the DNA, which provides a nucleation site for renaturation. That this phenomenon is due to a chemical cross-link may be demonstrated with S_1-endonuclease.[18] This enzyme, which specifically degrades single strand DNA, is virtually without effect on duplex DNA (Figure 2). DNA that has been treated with activated mitomycin C (reduction or protonation) or with carzinophilin A[36] is resistant to S_1-endonuclease.[18]

DNA Strand Breaks

Another, evidently common, lesion produced by antibiotics is that of DNA strand breakage. Three distinct kinds of single strand breaks (ssb) are recognizable:

Single Strand Breaks due to DNA Phosphotriester Formation and Hydrolysis. Treatment of PM2 DNA that has been relaxed by topoisomerase with 1-(2-chloroethyl)-1-nitrosoureas and certain aryltriazenes results in an immediate rise in fluorescence assayed at pH 11.8.[37] In contrast, dimethyl sulfate, which is known to alkylated DNA bases[38] but not phosphates, has no effect. The inference was that the anticancer agents were attacking the DNA phosphate groups, and the resulting phosphotriesters, while relatively stable under neutral conditions, undergo rapid basic hydrolysis.

RNA may be used to test whether this type 1 ssb and phosphate alkylation are related because RNA internucleotide linkages are less stable and the N-glycosylic bonds more stable than those in DNA.[39] Phosphotriesters of ribonucleotides are unstable over the entire pH range, presumably owing to the participation in the hydrolysis step by the β-hydroxy group on the sugar moiety.[39] Treatment of RNA with agents that produce type 1 ssb causes rapid degradation of the RNA determined by ultracentrifugation,[37] in contrast to agents

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that give rise to the following alternative lesion (type 11).

Single Strand Breaks due to Base Alkylation, Depurination or Depyrimidination, and Subsequent Hydrolysis of the AP Site. If the circular DNA treated with an antibiotic or antitumor agent is incubated in the assay medium at pH 11.8 used to detect type 1 ssb, then, after a time, a slower superimposed DNA scission is revealed.[37] The fact that agents such as $(CH_3)_2SO_4$ are especially effective leads one to suspect that this type of strand scission is a result of base alkylation, depurination or depyrimidination, and subsequent facile base hydrolysis of the AP site. This may be confirmed by employing endonuclease VI,[37] an AP site-specific enzyme that causes immediate breakage of the intermediate AP sites (Figure 4)[40] with concomitant rapid rise in the fluorescence values.

Free-Radical-Induced DNA Strand Breaks. A number of antibiotics, especially of the quinone type, have a reducing cofactor requirement and cause single strand breaks. These include mitomycin C,[18] mitomycin B,[28] streptonigrin,[20,41] saframycins A and C,[33] adriamycin,[42] bleomycin,[43] tallysomycin,[44] and neocarzinostatin.[45] Agarose gel electrophoresis may be used to detect both single and double strand breaks caused by such agents.[33] A unique advantage of the ethidium binding assay when used in conjunction with a supercoiled DNA (Figure 3) is that, by employing selective inhibitors (including certain cell protective enzymes), one may examine the chemical mechanism of the scission processes.[18,28,33,41,42,44]

DNA treated with the antibiotics mentioned above that have been reductively activated cause breaks that are uninfluenced by endonuclease VI. Neither do these antibiotics have an accelerating effect on RNA scission, indicating a mechanism different from type 1 or 11. This type of scission shows an oxygen dependence and is, however, inhibited by catalase and (depending on the antibiotics) by superoxide dismutase and by free radical scavengers such as mannitol, isopropyl alcohol, or sodium benzoate.[18,28,33,41,42,44] Since the denatured enzymes have no comparable inhibitory action, this inhibition is not due to nonspecific protein effects and therefore, where applicable, confirms the intermediacy of both $O_2^{-}\cdot$ and H_2O_2 while implicating that of the OH· radical. By invoking kinetic criteria (in conjunction with the OH· initiated generation and spin-trapping of the formyl radical produced from sodium formate), the generation of free OH radical was confirmed in the case of adriamycin, daunorubicin, streptonigrin, mitomycin C, and mitomycin B.[46]

In many of these cases, the obligatory semiquinone intermediate may be detected in vitro and in vivo and characterized by EPR in vitro by analysis of the hyperfine coupling.[25] In addition, the generation of H_2O_2 may often be detected in vivo.[47] These facts, together with the observation of the acceleration of DNA strand scission by traces of metal ions such as Fe(II).[18,25,31,41,42] or conversely its suppression by desferoxamine, are compatible with mechanistic Scheme I. Evidently

Scheme I

$$A + NADPH \xrightarrow{H^+} AH_2$$

$$A \xrightarrow[H^+,e]{\text{enzyme}} AH\cdot$$

$$AH_2 + O_2 \rightarrow AH\cdot + HO_2\cdot$$

$$AH\cdot + O_2 \rightarrow A + HO_2\cdot$$

$$HO_2\cdot \rightleftharpoons O_2^{-}\cdot + H^+$$

$$HO_2\cdot + HO_2\cdot \xrightarrow{SOD} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{\text{catalase}} H_2O_2 + O_2$$

$$CMX\text{–}Fe(III) + O_2^{-}\cdot \rightarrow CMX\text{–}Fe(II) + O_2$$

$$CMX\text{–}Fe(II) + H_2O_2 \rightarrow CMX\text{–}Fe(III) + OH\cdot + OH^-$$

$$OH\cdot + DNA \rightarrow \text{strand scission}$$

A = antibiotic; SOD = superoxide dismutase; CMX–Fe(III) = iron complexed with protein or ATP

these structurally diverse antibiotics, following reduction and reoxidation in vivo, give rise to reactive oxygen radicals including $HO_2\cdot$ (or $O_2^{-}\cdot$) and OH·. Depending upon the proximity of the particular antibiotic to a cellular macromolecule, this can give rise to membrane lipid peroxidation[48] and/or DNA damage.

The mechanism of the glycopeptide antibiotics bleomycin and tallysomycin, while similar in cellular effects to those of the quinone antibiotics, differs in detail in that, for example, the metal ion involved, Fe(II), is sequestered in the antibiotic.[49,50] The precise nature of the species giving rise to DNA strand scission in these cases is as yet unclear.

The selective cytotoxicity displayed by these antibiotics may be related to the fact that tumor tissue and certain organs (for animals and possibly humans) are significantly deficient in the protective enzymes, superoxide dismutase, catalase, and glutathione peroxidase.[51,52] A case in point is the severe dose-related cardiotoxicity of the anthracyclines that are known to cause significant cardiac lipid peroxidation.[48] By inference, this is likely to involve the mechanism outlined in Scheme I. An analysis of the structural factors controlling reactive oxygen species generation in the anthracyclines employing the ethidium binding assay and the independent measurement of hepatic microsomal oxygen consumption permitted a separation of antileukemic and cardiotoxic effects.[53] In the anthracyclines studied, a 20-fold amelioration of cardiotoxicity

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was effected with no sacrifice of antileukemic properties in that those anthracyclines that are less prone to enzymatic reduction and reoxidation are much less cardiotoxic.[53] Possible molecular origins of this phenomenon include differences in recognition characteristics of the anthracycline substrates by the metabolizing reductases.[53]

Intercalation and Bis Intercalation

The efficiency of intercalative binding[54] varies considerably depending on the additional functional groups (e.g., for hydrogen bonding) of a particular antibiotic resulting in different residence times. Examples of antibiotics that employ this kind of binding include the anthracyclines,[55] the glycopeptide antibiotics including bleomycin,[56] and the alkaloids ellipticine[2] and camptothecin.[57] A modification leading to more efficient binding is that of bis intercalation in which two such chromophores linked by a spacer of appropriate dimensions permits "non-nearest-neighbor" bis intercalation and is represented by echinomycin,[57] triostin,[58] Tandem,[59] luzopeptin,[60] and carzinophilin A.[36]

The changes in properties of the DNA resulting from intercalation may be detected by a number of physical techniques including viscosity,[59] and, quite conveniently, by the ethidium binding assay employing a topoisomerase.[61] Supercoiled DNA accepts more ethidium than relaxed DNA and is thereby unwound.[13] Consequently, treatment of supercoiled DNA with the topoisomerase (Figure 7) to the stage of complete relaxation results in a 30% decrease in bound ethidium. Prior treatment of the supercoiled DNA with an intercalating drug will unwind the DNA to a greater or lesser extent. Accordingly, subsequent treatment with the topoisomerase will have a proportionately smaller effect by which the intercalation of the antibiotic may be verified and quantified in that the unwinding angle may be calculated.[13]

Bis intercalators have a characteristic effect depending on the drug to phosphate residue (D/P) ratio by which they may be identified.[61]

Conclusions and Future Directions for Research

The utility of the approach of deducing particular chemical changes produced by antibiotics on intact native DNAs using enzymes is evident from the examples given. As more repair enzymes are isolated and their specific functions identified, it should be possible to refine the methods to detect specific effects. A major challenge is to determine the effects on these different antibiotic-induced lesions of proteins such as histones that are normally bound to cellular DNA. Ultimately, it may be possible by employing improved intercalative dyes and laser technology to detect individual chemical changes in DNAs by antibiotics in the intact cell.

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