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Mechanisms of in Situ Activation for DNA-Targeting Antitumor Agents

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I. Introduction

CC-1065 and the duocarmycins are the parent members of a potent class of naturally occurring antitumor antibiotics that exert their biological properties through the sequence-selective alkylation of DNA.[1−10] Although unreactive toward conventional nucleophiles at pH 7, the DNA alkylation reactions are exceptionally facile, proceeding in <1 h at 4−25 °C. Recent work defined the origin of this target-selective reactivity (shape-dependent catalysis) in which the forced adoption of a helical conformation upon binding minor-groove AT-rich regions disrupts the stabilizing vinylogous amide and activates the cyclopropane for nucleophilic attack.[11,12] In the course of these and related ongoing studies, we became interested in developing analogues of such natural products capable of alternative methods of activation. Thus, we sought to design systems where an intrinsically reactive species is formed from a comparatively stable precursor at or near the site of action. This strategy, known as in situ activation, can provide a means to selectively activate a compound in the presence of its biological target.

In situ activation, as we will discuss it, is distinct from the more general strategy of prodrug use. Prodrug systems are those where an inactive entity releases the active drug under appropriate conditions. This release can occur near to or distant from the site of action, and the released agent is typically less reactive than the prodrug itself. Although many of the in situ activation methods may be considered specialized subsets of prodrugs, some are not. Activation of CC-1065 and the duocarmycins is a beautiful example of one that is not, and its in situ activation does not involve a chemical change in the agent (i.e., prodrug). Rather, it is derived from a conformational change in the agent that occurs upon binding its target DNA that leads to activation only at the intracellular target site.

Several well-established methods have been discovered or developed for in situ activation of antitumor agents in the presence of their molecular targets. Many of these employ strategies that would not be easy to imagine emerging from de novo design efforts. Rather, most have been discovered through investigations of naturally occurring antitumor agents that defined the site and mechanism of action and provided a detailed understanding of the structural origin of the properties. In addition to shape-dependent catalysis as it applies to CC-1065 and the duocarmycins, these methods enlist oxidation, reduction, nucleophilic attack, elimination, photochemical activation, and metal complexation of suitable precursors to generate reactive species near or at the site of action. Herein we review examples of each of these strategies.

Although a great deal of attention has been directed at defining the chemical mechanisms of activation, little progress has been made in identifying the biological site at which the activation occurs. Known examples range from activation simply upon in vivo administration, within selected organs (e.g., liver), extracellularly, intracellularly, within specific intracellular compartments (e.g., nucleus), at the molecular target itself, or at sites defined spacially by exogenous activation. For this reason, it is often

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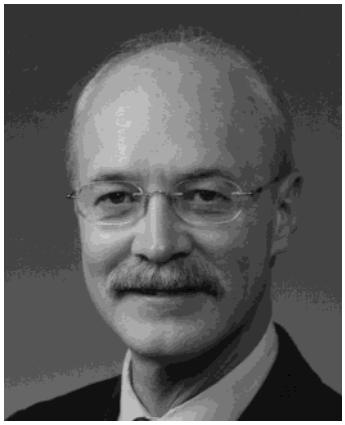

Born August 22, 1953, Dale L. Boger received his B.Sc. degree in Chemistry from the University of Kansas (1975) and Ph.D. degree in Chemistry from Harvard University (1980). Following graduate school, he returned to the University of Kansas as a member of the faculty in the Department of Medicinal Chemistry (1979–1985), moved to the Department of Chemistry at Purdue University (1985–1991), and joined the faculty at The Scripps Research Institute (1991 to present) as the Richard and Alice Cramer Professor of Chemistry. His research interests span the fields of organic and bioorganic chemistry and include the development of synthetic methodology, the total synthesis of natural products, heterocyclic chemistry, bioorganic chemistry, medicinal chemistry, the study of DNA–agent and protein–ligand interactions, and antitumor agents.

difficult to determine the relationship, if any, between activation and selective tumor cell cytotoxicity, both of which are properties of the agents discussed herein.

II. Shape-Dependent Catalysis

CC-1065 and the duocarmycins are exceptionally potent antitumor agents that derive their properties through a sequence-selective adenine N3 alkylation of DNA (Figure 1).[1−10] The differing reactivity of the natural products toward conventional nucleophiles (little or no reactivity) versus DNA (highly reactive) has been the subject of extensive study. This latter reactivity is the result of catalysis, which we have suggested is derived from a DNA binding-induced

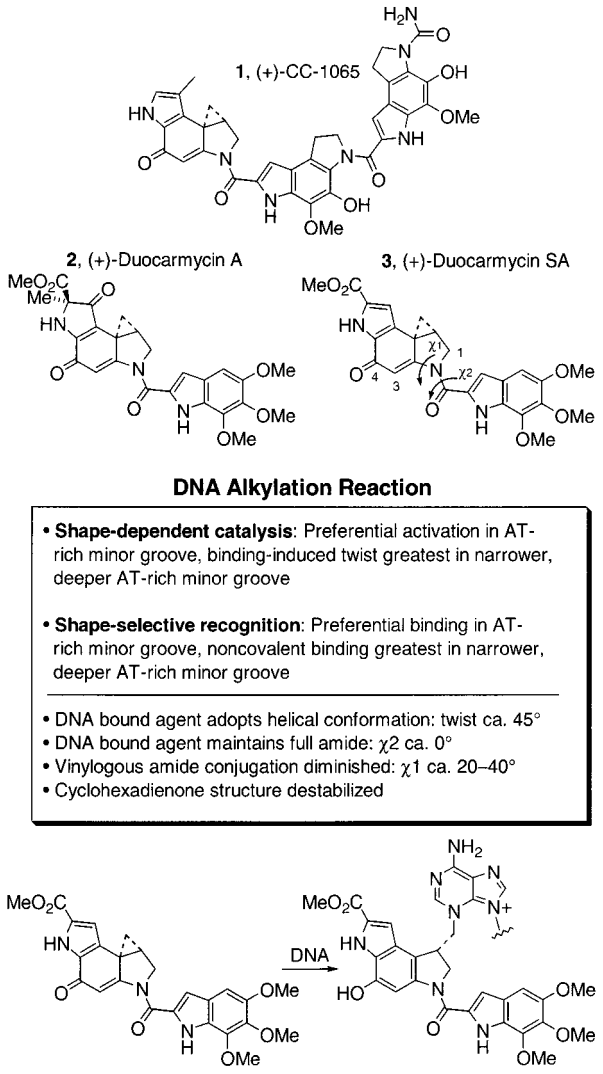

Figure 1.

conformational change in the agents which activates them for nucleophilic attack.[11,12] This conformational change twists the linking amide and disrupts the vinylogous amide conjugation stabilizing the alkylation subunit.

Insight into this source of catalysis for the DNA alkylation reaction was first described in the examination of reversed (**4**) versus extended (**5**) analogues of duocarmycin SA (Figure 2).[13] These studies established that the presence of the extended heteroaryl N2 amide substituent on the alkylation subunit conveys a special DNA alkylation reactivity that is independent of the alkylation sites and selectivity and that the reversal of the orientation of the DNA-binding subunits results in the complete reversal of the inherent enantiomeric DNA alkylation selectivity (Figure 2). Reversed analogues **4** displayed a substantial 100−1000-fold reduction in the rate of DNA alkylation, exhibiting rates similar to, but only 10-fold greater than, those of simple derivatives of alkylation subunits themselves (**6** and **7**, Figure 2). The modest 10-fold difference between the alkylation rates of **4** and **6** was attributed to the $CDPI_2$ enhancement of the DNA-binding affinity of **4**, whereas the larger 10^3-fold difference between **4** and **5** was attributed to the lack of binding-induced

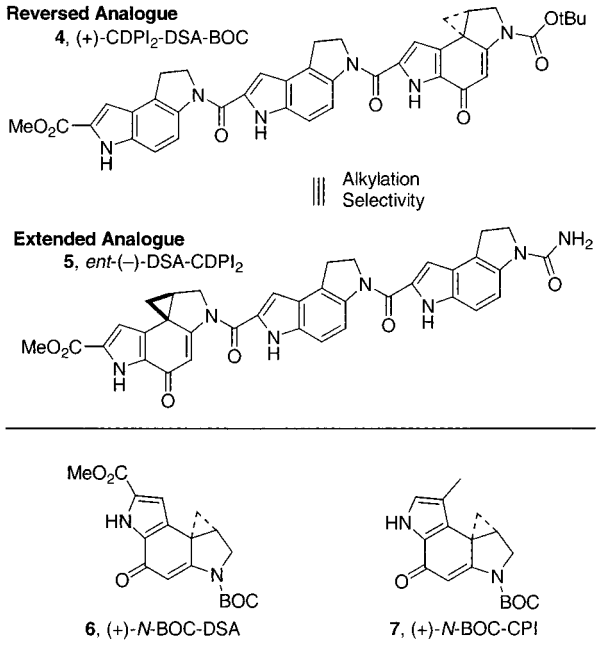

Figure 2.

catalysis for **4** derived from disruption of the stabilizing vinylogous amide.

These studies help shape a model which is based on the observation that **1**−**3** and simplified analogues **6** and **7** exhibit distinct alkylation selectivities[14−20] that are controlled by the AT-rich noncovalent binding selectivity of the agents and their steric accessibility to the adenine N3 alkylation sites.[1−5] Superimposed on this preferential AT-rich binding is the catalysis that we have suggested is derived from a binding-induced conformational change in the agents that twists the linking amide disrupting the stabilizing vinylogous amide conjugation activating the cyclopropane for nucleophilic attack.[11,12] Since the extent of this binding-induced conformational change is dependent on the shape of the minor groove, being greatest in the narrower, deeper AT-rich minor groove, we have come to refer to this as shape-dependent catalysis. This model accommodates the reversed and offset 3−4 or 5 bp AT-rich selectivity of the natural and unnatural enantiomers of **2** and **3**[17−20] and **1**[14−16] and requires that **6** and **7** exhibit alkylation selectivities distinct from the natural products. It offers a beautiful explanation for the identical selectivities of both enantiomers of **6** and **7** (5′-A<u>A</u>, 5′-T<u>A</u>), and the more extended AT-rich selectivities of **1**−**3** correspond nicely to the length of the agent, their bound orientation in the minor groove, and the size of the required binding region surrounding the alkylation site.[14−20] Further support for this model includes the established AT-rich noncovalent binding selectivity of the compounds,[21−23] their preferential noncovalent binding coincidental with DNA alkylation,[24] and the observations that relocation[25,26] or removal[27] of the C4 carbonyl, replacement of the cyclopropane with alternative electrophiles,[27] or use of exogenous sources of catalysis[28,29] do not alter the characteristic alkylation selectivity. The switch in the enantiomer alkylation selectivity of reversed versus extended analogues of the natural products coupled with the different but identical alkylation selectivities of enantiomeric sandwiched analogues confirmed that it is the noncovalent binding selectivity and not the alkylation subunit or alkylation site that is controlling the alkylation selectivity.[13,28−31] That catalysis is derived from a binding-induced disruption of the vinylogous amide is consistent with the requirement for a rigid, extended heteroaromatic N^2 amide substituent for catalysis,[13,30,31] the lack of catalysis when the linking amide carbonyl is removed,[32] structural correlations between vinylogous amide disruption and reactivity (10^4-fold reactivity increases),[32−36] and pH rate profiles of reactive alkylation subunits indicating that reactivity between pH 5 and 8 is not hydronium ion sensitive (not acid-catalyzed).[33−37] Further, this model explains subtle substituent effects on the rate of DNA alkylation that do not correlate with pH-dependent reactivity trends,[38−41] is consistent with observations that the DNA alkylation reaction is not acid catalyzed,[11] and that it does not rely on the DNA phosphate backbone for catalysis.[42] Structural studies of DNA-bound adducts have defined the required twist in the linking amide and have established consistent correlations with DNA alkylation rates.[43−45]

This unique mode of activation generates a highly reactive electrophile only at its site of action, within the minor groove positioned for nucleophilic attack by adenine. No chemical reaction is required for the activation; rather, the binding-induced twist in the linking amide is sufficient to accelerate the alkylation reaction >1000-fold. Hence, a highly reactive compound is generated only at its target site of action, and it is done so with high sequence selectivity.

III. Activation by Oxidation

A clinically important family of antitumor agents activated in situ by oxidation is the bleomycins (BLMs), the active components of Blenoxane. Bleomycin A_2 (Figure 3), the most abundant natural product in the clinically used mixture, has been studied in detail and has been found to induce single-strand (ss) and double-strand (ds) cleavage of DNA in the presence of iron and O_2.[46−50] The less frequent ds cleavage (1 for every 6−20 ss cleavages)[51−55] is believed to be responsible for the observed cytotoxic effects. Although early studies speculated that oxidative activation of iron−BLM takes place while it is

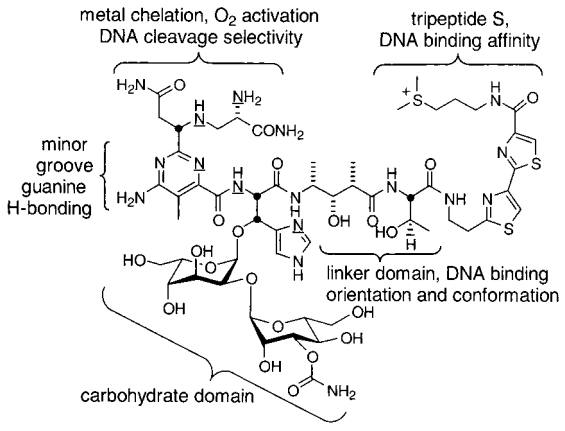

Figure 3.

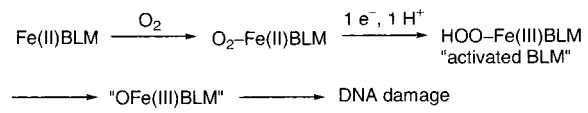

Figure 4.

bound to DNA,[46] subsequent studies have supported a sequence of events that entail metal chelation, oxidative activation, then DNA binding.[56−58] Iron−BLM is capable of being rapidly oxidized in the absence of its target to form reactive, yet respectively stable, intermediates (e.g., HOO−Fe(III)−BLM) responsible for DNA damage, although the intermediates decompose to inactive forms in the absence of DNA.[53,59,60]

Bleomycin can be separated into functionally independent domains: a metal-binding domain (including the disaccharide unit), a linker domain, and a DNA-binding domain. Of the possible sites of metal chelation, it has been proposed that the atoms underlined in Figure 3 are bound to iron in the active intermediates.[46,61,62] Notably, the bithiazole unit, a known metal chelator, can be excluded from metal binding by ^{13}C NMR[63] and fluorescence quenching studies.[64] The C-terminus tripeptide S subunit of BLM incorporating the bithiazole has been shown to be responsible for the majority of the DNA-binding affinity[63] by either minor-groove binding, partial intercalation, or both.[47,65] Finally, the N-terminus metal-binding domain controls the DNA cleavage selectivity.[66−68]

In situ activation of BLM involves reaction of BLM with both DNA and two essential cofactors: Fe(II) and O_2 (Figure 4).[69−72] Model BLM metal complexes bound to DNA have been characterized structurally using NMR, and key recognition contacts include a triplex-like H-bonding interaction between guanine and the pyrimidoblamic acid 4-aminopyrimidine anchoring the metal-binding domain in the minor groove.[61] Disagreement concerning the exact role of the bithiazole region and whether it binds the minor groove or intercalates has arisen from contrasting NMR[62] studies with Zn(II)−BLM[73−75] and HOO−Co(III)−BLM.[76−80] While both modes appear viable and lead to ss cleavage,[81] it is not yet clear whether the groove-binding model can generally provide the more biologically relevant ds DNA cleavage.[82]

Binding of Fe(II) to BLM is fast (reaction complete within 1 s)[83] and allows the formation of a ternary complex of Fe(II)−BLM with DNA. DNA does not inhibit the binding of Fe(II) and BLM, demonstrating the functional orthogonality of the DNA-binding and metal-binding domains.[83] Studies in DNA-free systems showed that O_2 binds to iron quickly ($t_{1/2} = 0.2$ s at 2 °C)[83] to generate O_2−Fe(II)−BLM, a diamagnetic species whose electronic structure is best formulated as O_2^-−Fe(III)−BLM[84] based on Mossbauer and other spectroscopic studies.[85]

O_2−Fe(II)−BLM is short-lived, exhibiting a half-life of 6 s at 2 °C, and it is reduced to the relatively stable HOO−Fe(III)−BLM,[84,86] a species referred to as "activated bleomycin".[83,87] This requires a single electron reduction,[88] and the identity of the intracellular reductant is not known. In cell-free systems, it has been suggested that the reducing agent is Fe(II)−BLM which, when uncomplexed with O_2, can supply one electron and generate Fe(III)−BLM.[59] This is supported by observations that the amount of DNA cleavage products is one-half that of the BLM consumed (stoichiometry of 2:1 for cleavage),[83] that one-half as much O_2 as BLM is consumed during cleavage,[59] and that DNA cleavage is inhibited by DNA (DNA sequesters BLM in solution, preventing a BLM−BLM interaction).[56,83] Furthermore, DNA cleavage is enhanced in the presence of organic reducing agents[72,89−92] capable of regenerating Fe(II)−BLM from Fe(III)−BLM, suggesting DNA cleavage by BLM can be catalytic. Several endogenous reducing agents, e.g., ascorbate, glutathione, and cysteine, have appropriate redox potentials to drive the conversion of Fe(III)−BLM to Fe(II)−BLM, indicating that a catalytic cycle can operate intracellularly.[88] It is also possible to form activated BLM from Fe(III)−BLM and H_2O_2,[83] and this method has been used to generate and study activated BLM anaerobically.

Activated BLM (HOO−Fe(III)−BLM) is the last detectable intermediate before DNA cleavage. Its consumption corresponds with the observance of DNA cleavage products.[83,86] Activated BLM is not stable, decaying in the presence of DNA with $t_{1/2} = 2$ min (0 °C)[83] to give Fe(III)−BLM and DNA cleavage products. In the absence of DNA, activated BLM decomposes to give a mixture of Fe(III)−BLM and other products.[59,60,93] The formation of cleavage products is initiated by abstraction of a C4′ H atom from the deoxyribose backbone of DNA.[94,95] Current mechanistic proposals for DNA cleavage begin with this event, although the identity of the BLM species abstracting the C4′ H atom has not been definitively characterized. Since activated BLM, HOO−Fe(III)−BLM, is the last detectable intermediate before DNA oxidation products are observed, it has been implicated as the abstracting species.[86,96] In addition, it is suggested that HOO−Fe(III)−BLM may undergo a heterolytic cleavage of the O−O bond to form a fleeting iron−oxo or iron−oxene which abstracts the C4′ H.[61,83,97,98] Regardless of whether H-atom abstraction occurs concomitantly with or after activated BLM heterolysis, the C4′ carbon radical is formed and directs the cleavage of DNA.

Two distinct pathways for cleavage have been defined (Figure 5).[47] When O_2 concentrations are low (Figure 5, left pathway), a single electron transfer from ribose to HO−Fe(IV)−BLM is believed to occur, generating a C4′ carbocation and HO−Fe(III)−BLM. The Fe(III)−BLM formed in this step is the observed BLM product of the overall reaction. The carbocation is trapped by water to form hemiketal **8**, which ring opens with free base release to give keto−aldehyde **9**, the "alkali labile" lesion.[94,99−102] Upon treatment with alkali, alkylamines, or hydrazine, **9** undergoes strand scission. Hence, the overall cleavage reaction at low O_2 concentrations results in one cleavage event for each iron−bleomycin complex. Kinetics and product ratios, however, indicate that ds DNA cleavage can arise from the action of one iron−BLM complex without dissociation from DNA.[51,54,55,103,104]

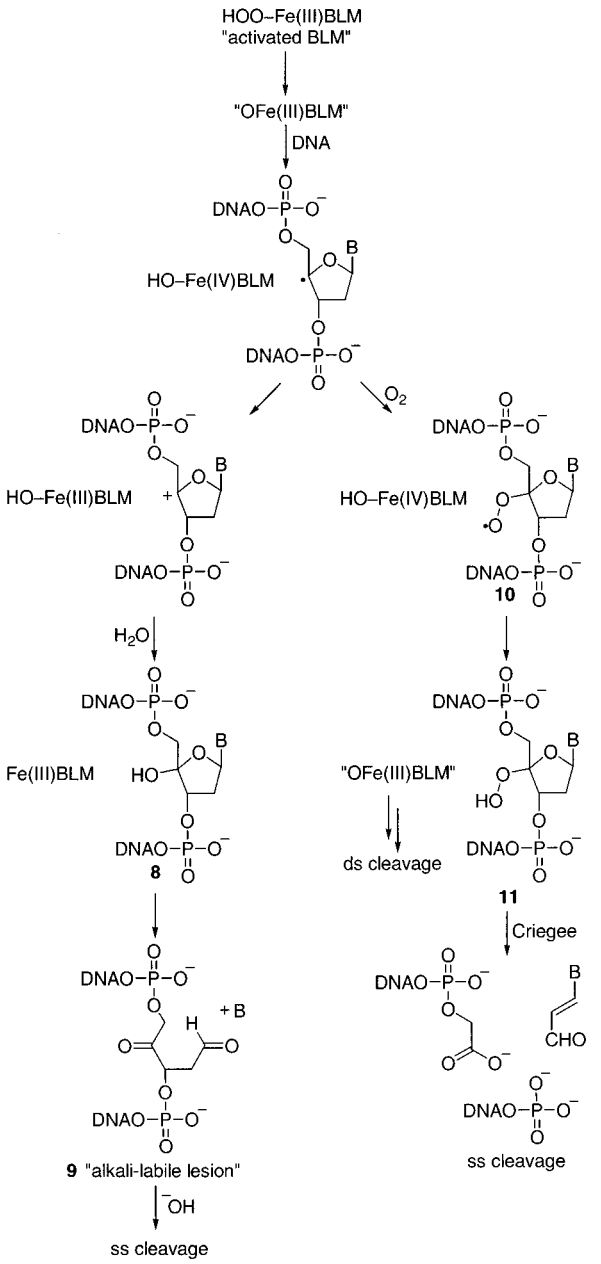

Figure 5.

While structural models to account for this observation vary,[81,82,105,106] there is a present consensus that ds cleavage can occur by the O_2-dependent cleavage pathway in Figure 5 (right pathway), which allows for one iron−BLM molecule to perform two strand cleavages. Under O_2-rich conditions, it is postulated that the same C4′ carbon radical is trapped by O_2 to give peroxy radical **10**, which abstracts an H atom from HO−Fe(IV)−BLM. This forms hydroperoxide **11** and generates the putative iron−oxo or iron−oxene. This species, proposed as the fleeting intermediate formed from the heterolysis of activated BLM and responsible for the initial H-atom abstraction, would then be capable of initiating a second cleavage after rearrangement or relocation to a different site. The hydroperoxide undergoes strand cleavage by a Criegee-type pathway[47] to generate a base propenal, a strand terminating in a 5′ phosphate, and a strand terminating in a 3′ phosphoglycolate. This proposed overall O_2-dependent cleavage pathway accounts for both the observed cleavage products and the ability for one iron−BLM complex to induce two strand scissions without dissociating from DNA.

Thus, key to the in situ activation of bleomycins are metal chelation and oxygen activation, both of which are required for observation of DNA damage and cleavage. At the site of its target, BLM is transformed into several unstable intermediates which initiate DNA cleavage. These features have been exploited in the development of the fundamental tools[107] of footprinting[108−110] and affinity cleavage[111] used to study DNA-binding agents and extended to new hybrid classes of antitumor compounds (e.g., synthetic distamycin−EDTA conjugates)[112] that exhibit sequence-selective DNA cleavage properties. Notably, the Fe(II)−EDTA-linked affinity cleavage reagents and hybrid structures rely on Fenton chemistry enlisting diffusible HO$^\bullet$ to oxidize and cleave DNA whereas BLM does not release a diffusible oxidant. Consequently, a more defined reaction with DNA is observed if such affinity cleavage agents or hybrid structures are prepared incorporating the BLM metal-binding domain.[113]

Finally, it is unlikely that the metal chelation and oxidative activation of BLM is responsible for its selective cytotoxic activity or its productive antitumor activity. Rather, it may be related to selective cellular penetration where BLM is not typically and effectively taken up by most cells or to differential intracellular trafficking or metabolism.[114]

Although not discussed in detail here since they are not regarded as antitumor compounds but rather as carcinogens, the aflatoxins (oxidative epoxide formation), polycyclic aromatic hydrocarbons (epoxide formation), and pyrrolizidine alkaloids (oxidative conversion of a dihydropyrrole to pyrrole) are activated in situ for reaction with DNA by alternative oxidation reactions.[115] Similarly, a number of additional, albeit synthetic, antitumor agents are oxidatively activated including cyclophosphoramide[116] and hexamethylmelamine.[117] They are among the first such drugs recognized to require oxidative metabolism and derive their biological properties from alkylating or cross-linking DNA.[118]

IV. Reductive Activation

The structural motif most common to reductively activated antitumor agents is the quinone.[119] Quinones exhibit reduction potentials on the order of endogenous reductases (cytochrome *c*, cytochrome b_5, xanthine), and their corresponding semi- and hydroquinones can be reoxidized back to quinones by O_2. Reductive activation is particularly well-suited for the treatment of hypoxic (O_2-deficient) tumors, where the bioreduction to hydroquinones is not easily reversed by endogenous oxygen. Once reduced, quinone antitumor antibiotics frequently generate alkylating agents by forming electrophilic quinone methides. Alternatively, quinone reduction can alter the electronic properties of the agent, triggering subsequent reactions. Finally, quinone reduction can lead to structural changes that induce cascade reactions ultimately responsible for DNA damage.

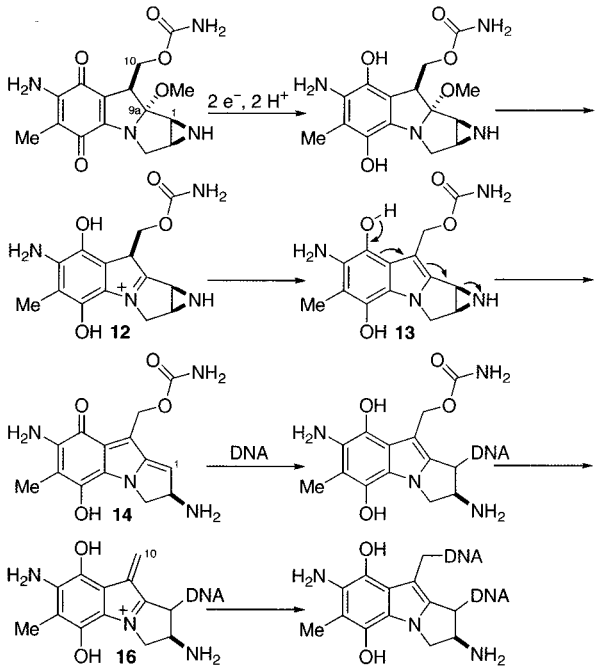

Figure 6.

Mitomycin C is a quinone antitumor antibiotic that exerts its biological activity through DNA alkylation and cross-linking. Nearly 40 years ago after demonstrating that metabolically activated mitomycin cross-links DNA,[120] Iyer and Szybalski proposed a mechanism for the activation and alkylation reactions which, after decades of experimental investigation, has been verified with few changes.[121] Their mechanism was based purely on structural considerations and chemical precedent. While its structure exhibits similarities to other quinone natural products, the mechanism of activation and cross-linking indicated in Figure 6 was without precedent at the time. Mitomycin is selective for hypoxic (O_2-deficient) cells characteristic of solid tumors,[122,123] and it is a classical example of an in situ activation mechanism that contributes to the selective cytotoxic properties of the molecule.

Mitomycin C itself is unreactive toward DNA at pH 7−8,[120] and surprisingly, neither it nor related compounds exhibit measurable or a characterized noncovalent binding with DNA.[124,125] However, upon enzymatic (old yellow enzyme,[126] *Clostridium kluyveri* diaphorase,[121] NADPH-cytochrome *c* reductase/NADPH,[127,128] xanthine oxidase/NADH,[127,128] DT-diaphorase[127,129]), metabolic (*Sarcina lutea* cell lysate,[121] rat liver microsomes[130]), electrochemical,[131,132] or chemical ($Na_2S_2O_4$,[121] $NaBH_4$,[121] H_2,[121,130] NO/HCO_2Na/$h\nu$[133]) reduction, efficient alkylation of DNA or trapping of nucleophiles is observed with DNA cross-linking proceeding at remarkable rates (<1 min).[121,134,135] In organic solvents, the reduction is reversible[136,137] and proceeds as two sequential one-electron transfers through the semiquinone to the hydroquinone with an overall reduction potential of −0.4 to −1.41 V depending on solvent and temperature.[131,132] The aqueous two-electron reduction potential of mitomycin was reported as −0.368[138] and −0.40[139] V vs SCE. Questions have been raised as to whether the in vivo activation cascade of mitomycin proceeds through the semiquinone[131,132,137,140,141] or hydroquinone form, although convincing evidence has been provided for the latter.[119,121,129,130,133,138,142−145] Both forms are capable of initiating the cascade, although each shows a strong solvent dependence: the hydroquinone requiring water[132,141] and the semiquinone requiring an organic medium.[132,133,141] In water, both one-electron and obligate two-electron reducing agents provide similar DNA alkylation product distributions suggesting a common intermediate.[142,144] Studies demonstrating the rapid disproportionation of the mitomycin semiquinone into the quinone and hydroquinone under aqueous anaerobic conditions indicate this common intermediate is the hydroquinone.[133] Under aerobic conditions, the semiquinone form is reoxidized to the quinone form by O_2 faster than it disproportionates, preventing its entry into the activation cascade and subsequent DNA alkylation.[146] This is proposed to form the basis for mitomycin's selective cytotoxicity toward hypoxic cells.[123] The one-electron reduction−reoxidation cycle which occurs in the presence of O_2 generates H_2O_2, $O_2^{-\bullet}$, and $HO^{\bullet}$, which are believed to be partially responsible for mitomycin's cytotoxic effects under aerobic conditions.[140,147−149]Although several enzymes can reduce mitomycin,[150] the identity of the biologically relevant reducing agent(s) is not known with certainty. Mounting evidence indicates it may be both the obligate two-electron reducing enzyme DT−diaphorase[129,150] and the one-electron reducing enzyme NADPH−cytochrome *c* reductase.[149,150] In addition to initiating the cascade which leads to DNA alkylation and cross-linking in cell-free systems,[144,145] DT−diaphorase has been implicated as the mitomycin reductant intracellularly. Human carcinoma cell lines which exhibit high levels of DT−diaphorase activity show greater susceptibility to mitomycin, and this susceptibility can be eliminated upon treatment with the DT−diaphorase inhibitor dicoumarol.[151,152] Additionally, cells which lack DT−diaphorase activity, when transformed with a DT−diaphorase-containing vector, become susceptible and produce mitomycin cross-linked DNA.[152] That DT−diaphorase is an obligate two-electron donor lends further support to the mechanism where the hydroquinone, and not the semiquinone, is responsible for mitomycin's activation and ensuing cytotoxicity.

Recent findings established that the bioreductive activation of mitomycin is an authentic chemical process that evolved in a microbial milieu to confer a selective advantage. A mitomycin-resistance gene *mcra* of the mitomycin-producing organism *Streptomyces lavendulae* has been discovered which produces MCRA, a flavin-containing oxidoreductase. MCRA oxidizes the mitomycin hydroquinone to the quinone with molecular oxygen acting as acceptor.[153] This gene confers resistance to mitomycin under aerobic conditions to recipient bacteria and transfected mammalian cells expressing MCRA.[154] The resistance protein has been shown to inhibit cross-linking of DNA by mitomycin hydroquinone under aerobic conditions in vitro, and the level of cross-linking inhibition correlates with the level of the mitomycin hydroquinone lost to reoxidation by

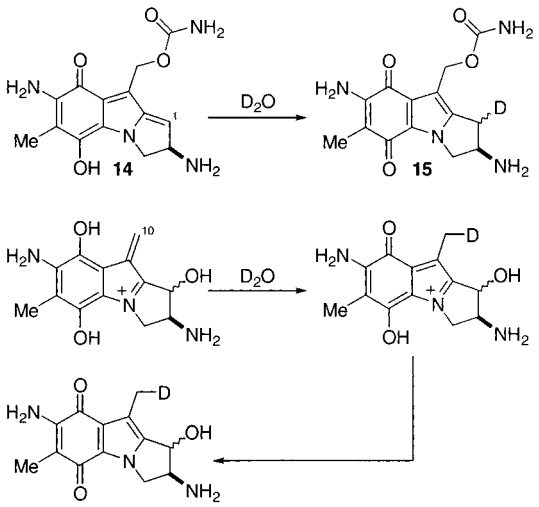

Figure 7.

MCRA.[134] These findings taken together reaffirm that the bioactive form of mitomycin is the hydroquinone and that DNA cross-linking by bioactivated mitomycin is the major cytotoxic event under aerobic conditions. Several mammalian heme peroxidases have been shown to similarly reoxidize the mitomycin hydroquinone, and it was proposed that this activity contributes to the lower sensitivity of aerobic tumor cells to mitomycin relative to hypoxic cells.[134,155]

Reduction of mitomycin releases the N4 lone pair of electrons from conjugation with the quinone via a vinylogous amide, enabling the expulsion of methoxide from C9a to form iminium ion **12**, and it is possible to trap this intermediate by treatment with Na_2SO_3[156] (Figure 6). Under anhydrous conditions, the methoxide elimination is not spontaneous,[136] suggesting protonation[121,133,142] prior to or concomitant with elimination. This initiates a cascade of reactions starting with formation of the indole **13** and subsequent conversion to the extended quinone methide **14**, with the release of aziridine ring strain. Support for the quinone methide **14** is available through indirect observation only and is derived from its dual electrophilic and nucleophilic reactivity. Intermediate **14** is the initial alkylating agent in the mitomycin cascade, trapping *O*-(H_2O, phosphate),[130] *S*-(xanthates),[157−159] and *N*-(aniline, DNA)[128,160−167] nucleophiles. The biologically relevant DNA alkylation proceeds on guanine residues at the 2-amino[128,162,163] and N7 positions[142,145] as observed in the monoadducts. The nucleophilic character of **14** was demonstrated by a protonation at C1, with the proton unequivocally derived from solvent,[168] to give the quinone product **15** (Figure 7). This quinone is usually a major byproduct of the reductive activation cascade of mitomycin.[130,140,167,169]

The second alkylating center of mitomycin is the iminium ion formed from the elimination of the C10 carbamate (Figure 6). Like the C1 quinone methide, **16** is unstable and exhibits dual electrophilic and nucleophilic character at C10. C10 is the second site of alkylation in mitomycin cross-link formation, forming a bond with the 2-amino group of guanine.[160] C10 can also be protonated, and deuterium-labeling studies show that the proton is derived from solvent

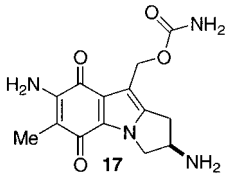

Figure 8.

(Figure 7).[132,170] The C1 alkylation always precedes reaction at C10. This was shown unequivocally for the DNA alkylation reaction when the purified mitomycin C C1 DNA monoadduct was reactivated with $Na_2S_2O_4$ and provided cross-linked products.[171] Monoadduct formation at C10 has been observed[145,172] but only after protonation of the C1 quinone methide followed by rereduction of the quinone, elimination of the carbamate, and nucleophilic attack.

The reduction of mitomycin can be autocatalytic, and stoichiometric DNA alkylation and nucleophilic additions have been observed with substoichiometric amounts of reducing agents.[126,133,141,171] After reduction has initiated the activation cascade of one molecule, the reduced mitosene **13** and its alkylation products are capable of reducing a second molecule of mitomycin in solution. Since the quinone form of **13** acts only as a monoalkylating agent,[167] monoadduct/cross-link product ratios are affected by the extent to which this pathway operates, determined by the varying rates of reduction by different reducing agents.[142,160] However, it has been shown that most enzymatic and chemical reductants give identical product ratios under comparable conditions.[142]

Both inter- and intrastrand[160,165] cross-links are observed when mitomycin reacts with DNA, although the former predominates. As mentioned above, the ratio of bisadduct formation (cross-linking) to monoadduct formation depends on the rate of reduction, but the sequence selectivity of these reactions does not. The mitomycin C interstrand cross-link is "absolutely specific to the 5′-CG duplex sequence"[173−177] with the agent spanning the distance between the two strands across a one base-pair step in the minor groove.[164] The intrastrand cross-link is specific to 5′-GG.[160,165] The observation that 2,7-diaminomitosene (**17**, Figure 8), which is incapable of DNA alkylation, binds to DNA nonselectively makes the exquisite selectivity of cross-link formation even more impressive.[124] Models accounting for this selectivity, largely verified through NMR structural and modeling studies,[160,164,178] propose that most of the cross-linking sequence selectivity is derived during the first alkylation event, which is quite selective.[175,176] Central to this selectivity is a hydrogen bond between the 2-amino group of guanine on the strand opposite the site of alkylation and the C10 oxygen of mitomycin (Figure 9). This key interaction is inferred from studies in which the 2-amino group of guanine is removed (inosine substitution), which results in greatly diminished alkylation.[176] A second contribution to the selectivity of the cross-link stems from the preferred orientation of the covalent monoadduct in the DNA minor groove, in which it is poised to form the cross-link without significant structural reorganization of the DNA. This single-orientation conformer was observed in high-

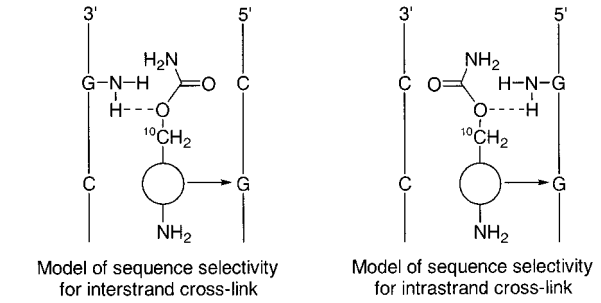

Figure 9.

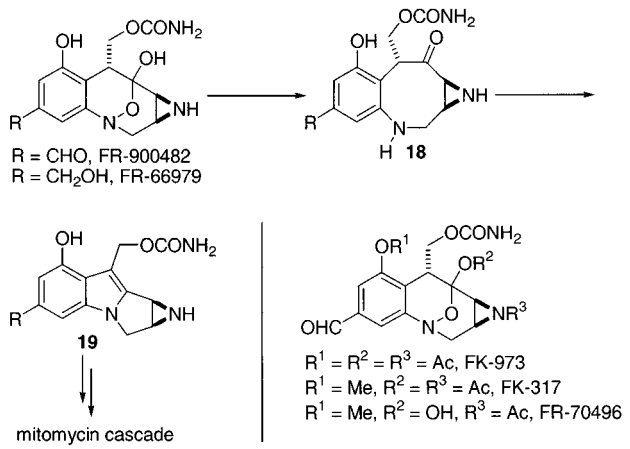

Figure 10.

resolution NMR studies of oligonucleotide mitomycin monoadduct complexes[179] and supported by molecular modeling.[176]

Mitomycin's in situ activation begins with selective reduction in the target hypoxic cells and follows a cascade of reactions to form two reactive intermediates, both of which alkylate DNA sequence selectively. A recently discovered class of natural products, exemplified by FR-900482[180] and FR-66979,[181] generates the same types of reactive intermediates in their in situ reductive activation cascade[182,183] as shown in Figure 10. In this case, the key reduction involves cleavage of an N−O bond, leading to the fused eight-membered ring ketone **18**. Attack of the arylamine on this ketone, followed by elimination of water leads to **19**, a mitosene analogous to that formed in the activation cascade of the mitomycins (**13**).[184,185] Thus, it is not surprising that both FR-900482 and FR-66979 form interstrand cross-links in DNA with the same 5′-CG sequence selectivity as mitomycin.[183,185] The quinone methide formed from **19** by opening of the aziridine, analogous to that observed in the mitomysin cascade (Figure 6), does not have dual electrophilic and nucleophilic reactivity at C1 and would not be expected to be protonated at C1. The high efficiency of FR-66979 as a cross-linking agent[184,185] may be due to the lack of this side reaction in the activation cascade. Recently, other biologically active members of this structural class, which are believed to be activated by the same mechanism, have been disclosed (FK-973, FK-317, FR-70496).[186] Because the activation cascade does not involve semiquinone intermediates, this class of agents is incapable of generating damaging oxygen-derived

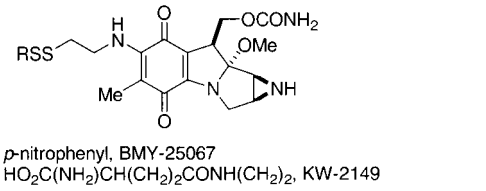

Figure 11.

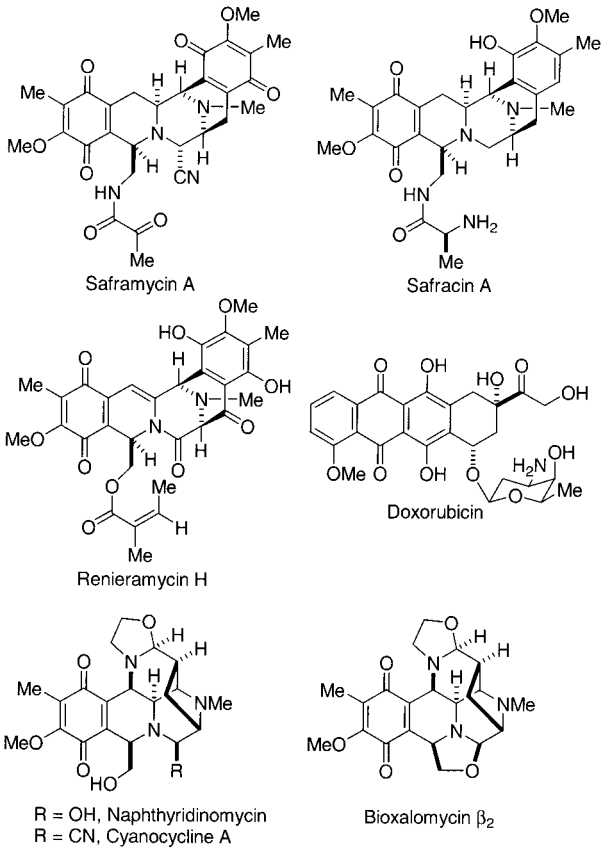

Figure 12.

radicals, and this may account for the comparatively lower toxicities of FR-900482 and FR-66979 under aerobic conditions.[187] Importantly, these agents have been shown to provide both DNA cross-linking adducts and cross-links between DNA and bound proteins (HMG I/Y proteins).[118,188−191] The intrinsic complexity, practical utility, and clinical success of the mitomcyin/FR-900482 and FR-66979 activation cascade have spawned efforts to apply it in designed systems as well. Semisynthetic mitomycin analogues BMY-25067[192] and KW-2149[193] incorporate unnatural side chains at the 7-amino position which alter the activation pathway (glutathione attack instead of bioreduction)[194,195] while retaining the characteristic mono- and bisalkylation properties of the parent compounds (Figure 11).

Additional natural product antitumor agents that form alkylating quinone methides upon quinone reduction include the saframycins and related renieramycins and safracins,[196,197] the anthraquinones including doxorubicin, and naphthyridinomycin and the related cyanocyclins and bioxalomycins (Figure 12).[118] Recently, the DNA cross-linking of bioxalomycin α_2, the hydroquinone form of bioxalomycin β_2, at 5′-CG sites (G−G cross-linking in the minor

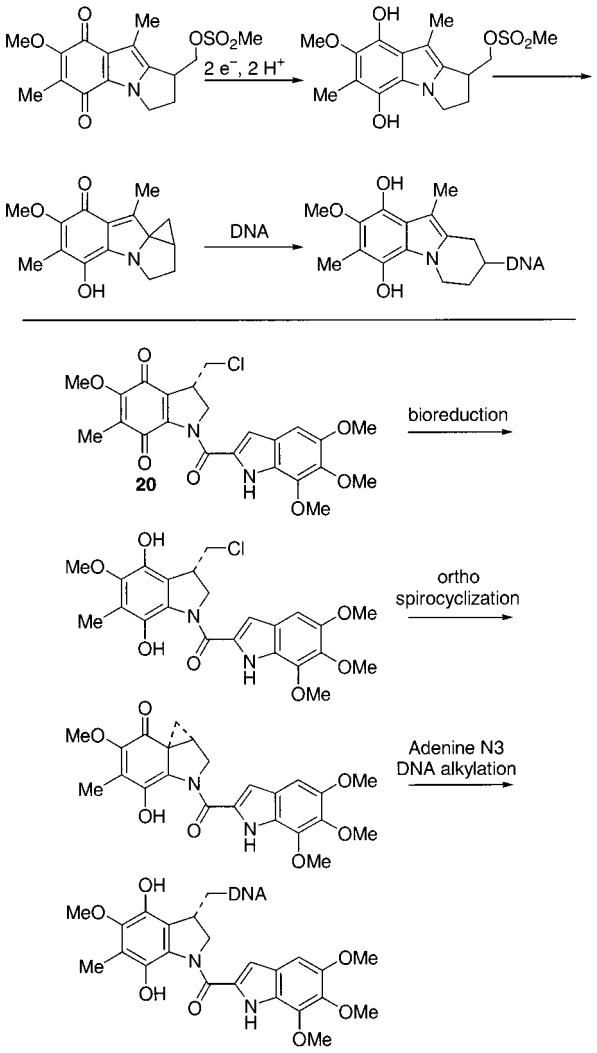

Figure 13.

groove) has been established.[198] Like mitomycin C, these compounds are also reduced via single-electron transfer generating semiquinones which can either react directly with cellular targets or form damaging oxygen radicals.[118,199−203]

Hybrids designed to incorporate the reductive activation of mitomycin into the framework of CC-1065 and the duocarmycins have been described. Two approaches, involving cyclopropane formation at the corresponding mitomycin C1 and C10 positions, have been examined. In the first example, the aziridine of mitomycin is replaced with methylene methylsulfonic acid at C1 (Figure 13, top).[204] The one-carbon homologation precludes extended quinone methide formation upon reductive activation of this agent. Instead, cyclopropane ring formation occurs, and it was shown to alkylate DNA at the N7 position of guanine. A more recent example of this strategy takes advantage of reactivity at what would be C10 of mitomycin (Figure 13, bottom).[26] In this case the carbamate is replaced with chloride and, more importantly, the methoxy substituent at what would be mitomycin position C9a is removed. This makes it impossible for this compound to form the iminium ion that is the second alkylating species in the mitomycin reductive activation cascade. Instead,

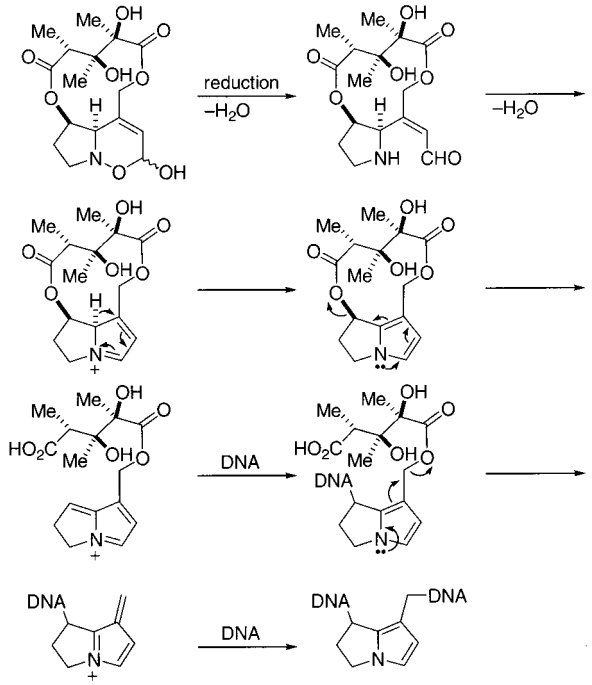

Figure 14.

spirocyclopropane formation is induced generating a species analogous to *iso*-CBI congeners of duocarmycin SA, whose duocarmycin-like DNA alkylation properties were well documented.[25] Consistent with the design, **20** was found to alkylate DNA only upon quinone reduction and to do so with a sequence selectivity indistinguishable from the natural product duocarmycin SA. Additionally, **20** was shown to be a substrate for DT−diaphorase and to have greater potency in a lung cancer cell line exhibiting high DT−diaphorase activity. Thus, **20** was found to behave as a hybrid of the two natural products constituting a duocarmycin analogue subject to a mitomycin-like in situ reductive activation.

Similarly, clever ways to design a reductive activation trigger for pyrrolizidine alkaloids capable of DNA cross-linking have been detailed[205] mimicking the reductive N−O bond cleavage of FR-900482 and FR-66979 (Figure 14). In part, this was based on the natural product precedent of monocrotaline and other pyrrolizidine alkaloids which are carcinogens that form bisalkylating agents upon oxidation.

Aziridinylbenzoquinones[206,207] (e.g., AZQ, DZQ) represent a much more significant departure from the in situ activation cascade of the mitomycins, in which only the initial steps are retained (Figure 15).[208,209] These synthetic antitumor agents incorporate two initially unreactive nitrogen mustard moieties into a quinone scaffold and rely on reactivity differences between the quinone and reduced forms for in situ activation. After an initial reduction, which occurs selectively in hypoxic cells, the nitrogen mustard lone pair of electrons is released from vinylogous amide conjugation with the quinone.[169,210,211] The resulting increase in electron density on the nitrogen atoms permits their pH 7 protonation and the subsequent ring-opening alkylation reaction of the aziridine. Modulation of the aziridine reactivity has been achieved by varying the substitutents on the quinone

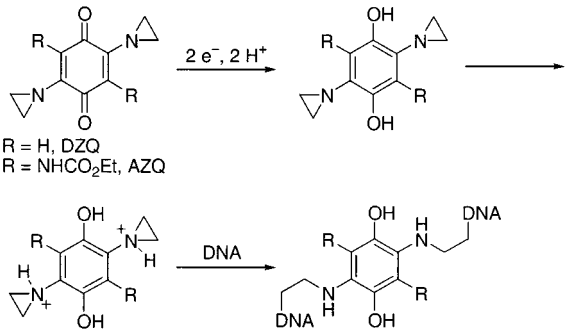

Figure 15.

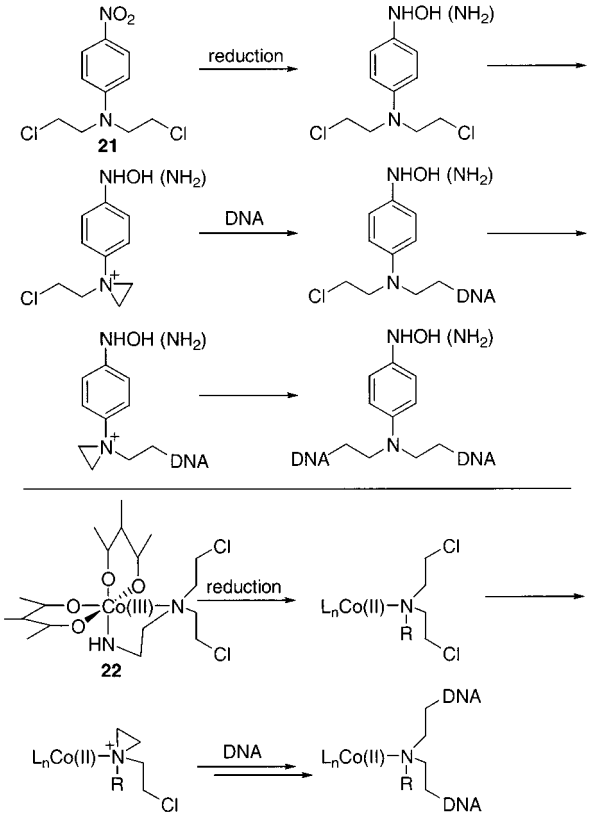

Figure 16.

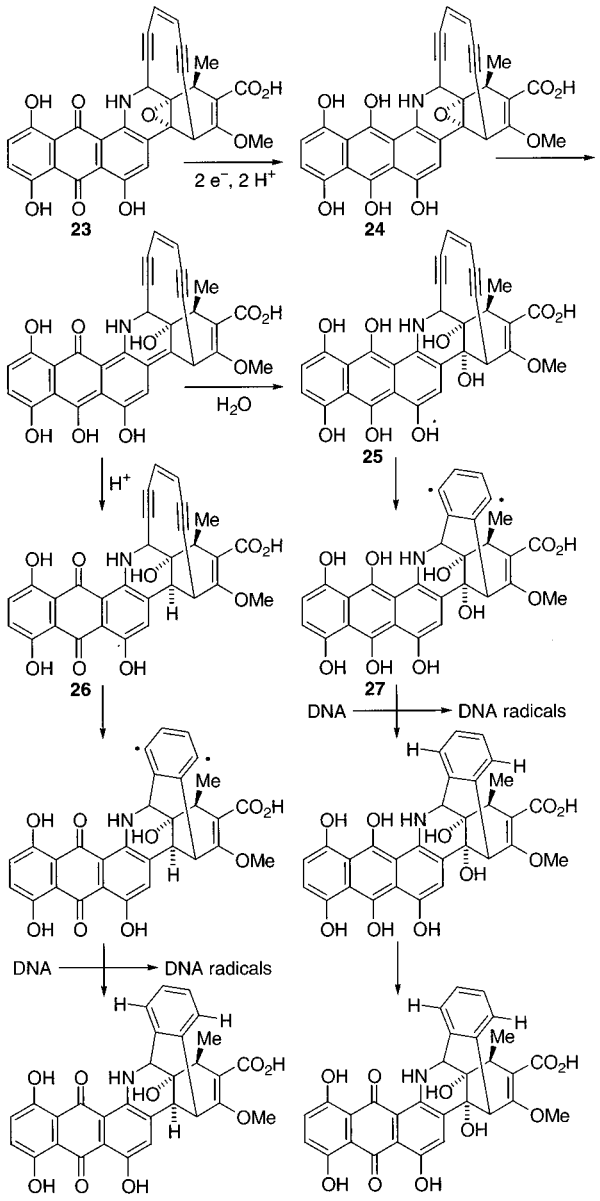

Figure 17.

ring, affecting the reduction potential and allowing its selective in situ activation in hypoxic cells.

Another series of agents that rely on both reduction and nitrogen mustard formation for in situ activation are shown in Figure 16. Like AZQ, each relies on an increase in electron density on nitrogen to induce aziridine formation. In contrast to a quinone reduction, they rely on a reductive functional group interconversion to modulate the nitrogen electron density. Reduction of nitroaromatic **21**[212−214] or the Co complex **22**[215−217] liberates amines that are more electron rich and readily form the electrophilic aziridine.

A more dramatic extension of reduction-initiated quinone methide formation is exhibited by dynemicin (Figure 17). Dynemicin A (**23**), the first of this class of natural products to be isolated, incorporates an anthraquinone-linked enediyne into a structure containing a key epoxide.[218,219] This rigid polycyclic structure keeps the alkynes spacially separated, preventing a Bergman cycloaromatization which generates DNA-damaging radicals. Upon reduction of the quinone, a cascade is initiated which brings the two triple bonds into close proximity permitting cycloaromatization via a Bergman cyclization.[220−223] Formation of the hydroquinone **24** is followed by epoxide ring opening via extended quinone methide formation. Like the extended quinone methide formed in the activation cascade of mitomycin C, it exhibits both electrophilic and nucleophilic character.[224−226] It is either trapped by water to form hydroquinone **25** or protonated to give quinone **26**.[226] In either case, epoxide opening induces cycloaromatization and diradical formation. These aromatic radicals, as in the case of other enediynes (see below), are responsible for single- and double-strand DNA cleavage through processes involving H-atom abstraction.[224,227,228] In the case of **27**, the resulting hydroquinone is oxidized by endogenous O_2 to the observed quinone product. In the case of dynemicin A, in situ activation is initiated by a quinone reduction analogous to mitomycin C but leads to very different unstable biradical

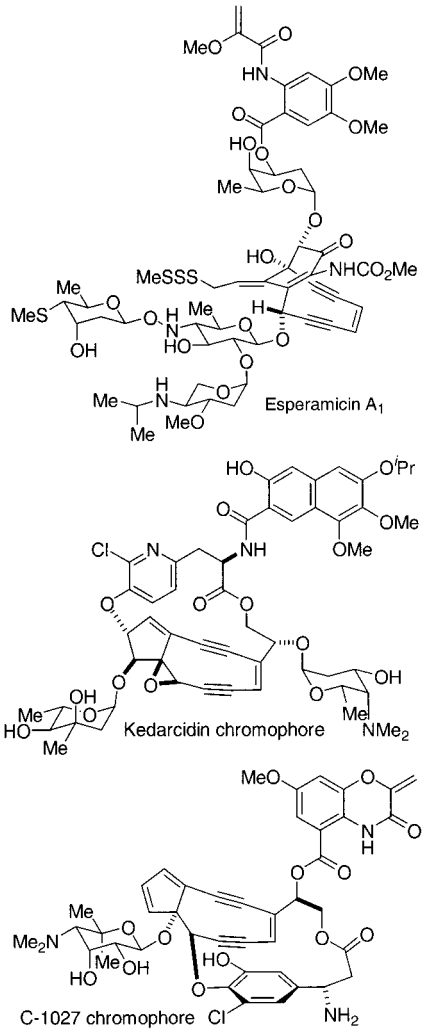

Figure 18.

intermediates that are responsible for the observed biological effects.

V. Activation by Nucleophilic Addition

Whereas dynemicin A is activated by a reductive pathway, most enediyne activation cascades are triggered by a nucleophilic addition.[229,230] This applies to calicheamicin,[231,232] esperimicin,[233] kedarcidin chromophore,[234] neocarzinostatin (NCS) chromophore,[235,236] C-1027 chromophore,[237] and maduropeptin chromophore[238] (Figure 18) and is detailed for NCS chromophore and calicheamicin in Figures 19 and 20. In the case of NCS chromophore,[236] attack of a thiol nucleophile at C12 results in ring opening of the pendant epoxide with formation of a highly strained cumulene (Figure 19, top).[239,240] The extended cumulene−ene−yne is believed to undergo a Myers cycloaromatization[241] with generation of diradical **28**,[242] which abstracts H atoms from DNA leading to strand cleavage and formation of **29**, the observed NCS product.[243−249] Interestingly, the NCS chromophore may also be activated by a general base-catalyzed intramolecular addition reaction in the absence of nucleophilic thiols[250,251] (Figure 19, bottom). When incubated with DNA containing bulged structures, this base-catalyzed reaction[252,253] results in site-

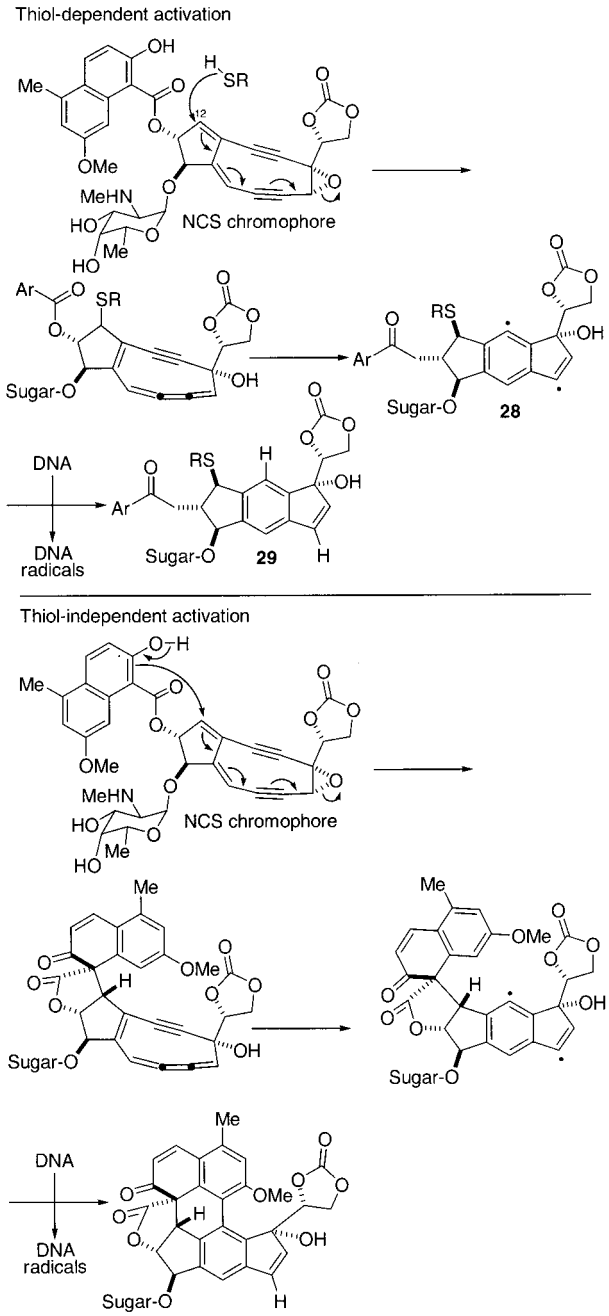

Figure 19.

specific cleavage. The nucleotide requirements of the bulge,[254] the structures of the NCS chromophore-derived products,[255,256] and a detailed mechanism for cleavage[253] have been elucidated by Goldberg and coworkers. Among the enediyne antibiotics disclosed to date, only NCS chromophore has such an alternative mechanism of activation available.

Calicheamicin is also activated in situ by attack of a thiol,[257,258] although attack occurs at the trisulfide and results in the release of the calicheamicin thiolate (Figure 20). This anion undergoes a Michael addition to the bridgehead α,β-unsaturated ketone to form the dihydrothiophene **30**. The accompanying change in hybridization at C9 ($sp^2 \rightarrow sp^3$) triggers a Bergman cyclization[223] to generate biradical **31**. This biradical abstracts H atoms from DNA, resulting in strand cleavage.

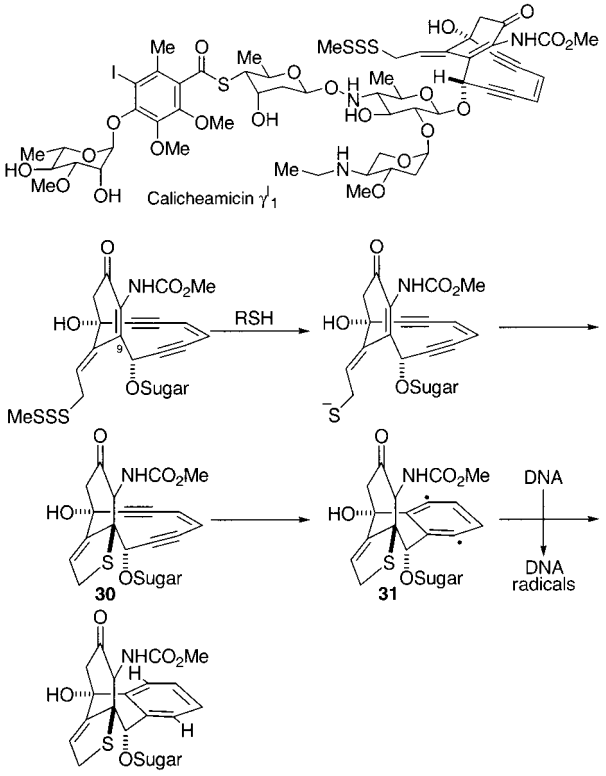

Figure 20.

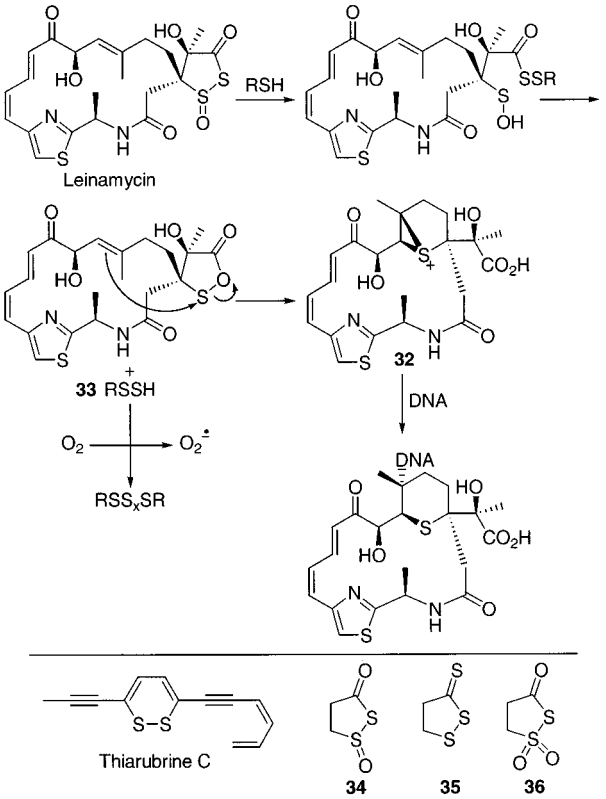

Figure 21.

NCS chromophore, maduropeptin chromophore, kedarcidin, and C-1027 were isolated along with companion proteins and are highly reactive upon their release. Although this protein-binding stabilization was thought to arise simply from protective shielding from solvent and cellular nucleophiles, evidence is emerging which suggests that the protein-bound conformations and/or interactions may additionally stabilize the endiynes and that the proteins themselves may serve to deliver the endiynes to intracellular target DNA.[259−263]

Finally, although the enediyne activation has been typically associated with DNA damage by H-atom abstraction and DNA cleavage, adducts of NCS chromophore and C-1027 have been observed under anaerobic conditions.[264−271]

A structurally distinct class of natural products which target DNA and are activated by nucleophilic attack include leinamycin and thiarubrines (Figure 21), which contain S−S bonds. In the case of leinamycin,[272] nucleophilic attack by thiol,[273] cyanide, or phosphine[274] at the sulfenyl sulfur atom triggers a cascade of reactions releasing the hydrodisulfide of the attacking thiol (**33**) and generating the electrophilic episulfonium ion **32**. This intermediate alkylates DNA at the N7 position of guanine,[272,275] leading to single-strand cleavage and release of the leinamycin−guanine adduct.

In addition to this mode of DNA damage, the hydrodisulfide product **33** of the activation cascade has been implicated in the production of DNA-damaging oxygen radicals. This has been observed with the natural products leinamycin and thiarubrine C[276] as well as in simplified systems containing only the 1,2-dithiolan-3-one 1-oxide (**34**),[277] 1,2-dithiole-3-thione (**35**),[278] and 1,2-dithiol-3-one 1,1-dioxide (**36**)[279] heterocycles. In the presence of O_2, the intrinsically unstable hydrodisulfides **33** oxidatively dimerize to form polysulfides with the generation of DNA-damaging superoxide.

An additional class of antitumor agents activated in situ by nucleophilic attack is exemplified by the illudins[280,281] and the synthetic analogue hydroxymethylacylfulvene (HMAF, Figure 22).[282] These fungal metabolites alkylate amines in DNA by nucleophilic attack on the cyclopropane ring of an activated intermediate. As in the case of the enediynes and leinamycin, formation of this unstable species is initiated by nucleophilic attack of a thiol. The ensuing activation cascade and DNA reactivity have also been proposed for the structurally similar antitumor antibiotics myrocin C[283] and the potent carcinogen ptaquiloside.[284,285] However, both myrocin C and especially ptaquiloside require a key penultimate elimination reaction to generate the analogous activated cyclopropanes. Since the activation mechanism of myrocin C has not yet been verified as related to its biological properties and that of ptaquiloside generates a carcinogen, this has not been treated as a separate mechanism of activation (elimination reaction).

At neutral pH, the illudins and HMAF are unreactive toward oxygen, halogen, and nitrogen nucleophiles, including DNA.[281] However, a facile Michael addition to the α,β-unsaturated ketone is observed upon treatment with thiols (e.g., methylthioglycolate, cysteine, glutathione),[282,286] generating reactive diene **37**. The cyclopropane in this intermediate is activated for nucleophilic attack upon protonation of the sensitive tertiary allylic alcohol leading to elimination of water with formation of an aromatic system. In DNA-

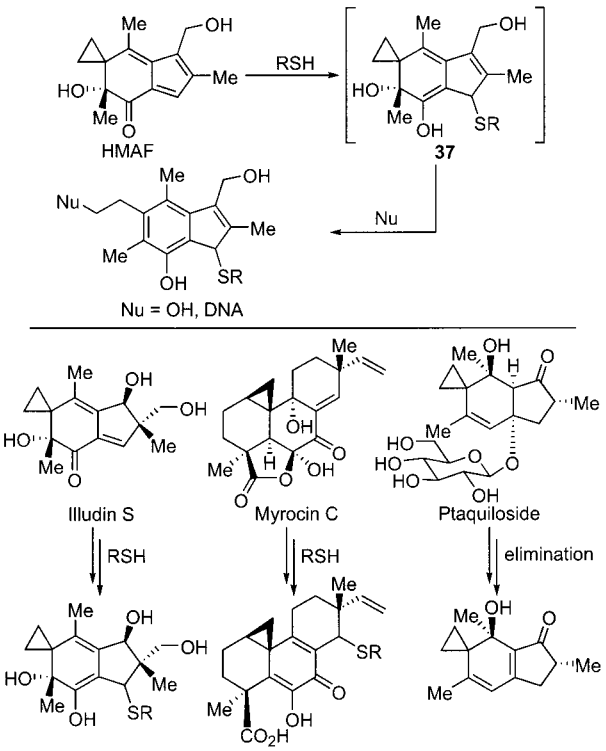

Figure 22.

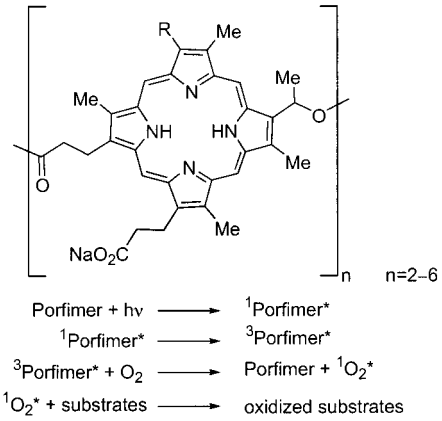

Figure 23.

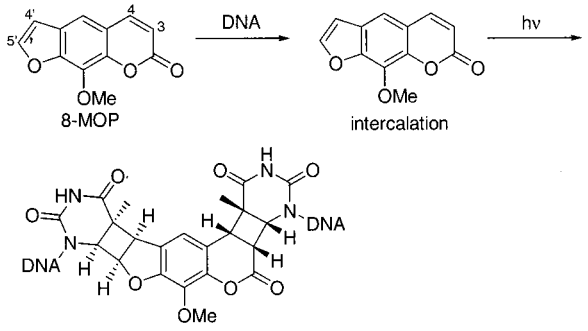

Figure 24.

free systems, the cyclopropane undergoes ring opening with attack of water.[281] Although neither the illudin−DNA nor the HMAF−DNA adducts has been isolated, studies indicate that the illudin target is DNA.[287] Indirect evidence for monoadduct formation and single-strand scission in the reaction of HMAF with cellular DNA has been reported.[288] The inhibition of primer extension by *Taq* polymerase (PCR assay) suggests monoadducts, while sedimentation studies in sucrose gradients which document a gradual shift toward shorter lengths of DNA indicate single-strand cleavage events without DNA cross-linking. Whereas these results suggest facile single-strand cleavage following alkylation, HMAF did not relax supercoiled DNA in a gel shift assay.[288] In addition, the close structural relationship with the well-documented ptaquiloside DNA alkylation reaction strongly suggests this mode of action. In the case of ptaquiloside, attack on the cyclopropane of the activated diene occurs primarily at N3 of adenine as well as N7 of guanine, resulting in unusually facile, room-temperature single-strand cleavage reactions.[284,285]

VI. Photochemical Activation

Photochemical activation in situ constitutes the targeting of certain tissues with light. This strategy requires administration of the photoactive compound followed by irradiation of the area to be treated with UV light.[289,290] In this sense, photochemical activation differs from the other strategies of in situ activation since it does not depend on endogenous activation but rather an external stimulus.

Compounds approved for clinical use are of two classes: porphyrins[289,291] and furocoumarins.[290] In both cases, the agents are administered and the affected tissues are irradiated to activate the compound locally. The unstable intermediates are short-lived and do not diffuse into surrounding healthy tissue.

Of the many porphyrins synthesized and evaluated, porfimer (Photofrin) has been approved for the photodynamic treatment of esophageal and endobronchial cancers (Figure 23). This mixture of oligomeric porphyrins based on hematoporphyrin is reactive toward 630 nm laser light, generating singlet excited-state porfimer.[292] Through nonradiative intersystem crossing, the singlet converts to the more stable triplet which is sufficiently long-lived to undergo intermolecular reactions, most importantly with oxygen.[293,294] The singlet oxygen generated in this reaction is believed to be the damaging agent, oxidizing intracellular targets nonspecifically and generating superoxide and hydroxyl radicals, which are also damaging.[295] Although porfimer is injected intravenously and is distributed throughout the body, selective generation of singlet oxygen in a tumor is achieved by irradiation with a fiber optic diffuser inserted through an endoscope.

The selective activation of 8-methoxypsoralen (8-MOP, Uvadex, methoxsalen), the only clinically approved furocoumarin used in photochemotherapy, utilizes a different strategy (Figure 24).[290] It is useful in the treatment of cutaneous T cell lymphoma, in which cancerous T cells proliferate in the blood. Instead of bringing light to the tumor via fiber optics, use of this agent involves bringing the tumor cells to the light. 8-MOP is administered orally, and then blood is drawn and leukocytes (including T cells) are irradiated with UVA (320−400 nm) light. The treated

leukocytes are then recombined with plasma and red blood cells and reinjected into patients.

The chemically active species in 8-MOP treatment differs from the porphyrins. Singlet oxygen is not generated, and 8-MOP itself is both the chromophore and the damaging species. 8-MOP is a DNA intercalator, which has been shown to bind DNA noncovalently with a weak affinity ($K_d = 10^{-4}$ M).[296] Nonetheless, upon irradiation with near UV light, 8-MOP forms [2 + 2] cycloadducts with adjacent bases. Cycloaddition occurs across both the C4′−C5′ and C3−C4 double bonds of 8-MOP, and both mono-[297,298] and bisadducts (cross-links)[299] are isolated. Reaction occurs most commonly with thymine C5−C6 double bonds, forming products similar to thymine cyclobutane dimers. Although 8-MOP has a relatively low affinity for DNA, it is very selective in its binding. Of the eight possible monoadduct stereoisomers and the 64 possible bisadduct (cross-link) stereoisomers, only *cis-syn* products are isolated.[299] This is believed to be a consequence of 8-MOP intercalation which maximizes the overlap between the psoralen and adjacent pyrimidine bases.

T cells containing cross-linked DNA do not survive treatment, but an unexpected benefit was observed upon reinjection of the treated cells into patients.[290] The injected cells elicit a strong immune response which is not limited to T cells containing DNA cross-links. In fact, the response includes recognition and destruction of malignant T cells which have not been irradiated with light.

VII. Activation by Metal Complexation

Recently, we reported a new means of in situ activation for a novel class of DNA alkylating agents.[28,29] Methyl 1,2,9,9a-tetrahydrocyclopropa[*c*]-pyrido[3,2-*e*]indol- 4-one-7-carboxylate (CPyI, **38**) contains a unique 8-ketoquinoline structure which provides a tunable means to effect activation via selective metal cation complexation (Figure 25). This activation promotes a DNA minor-groove adenine N3 alkylation in a manner analogous to that of CC-1065 and the duocarmycins, upon which CPyI was based. A study of CPyI activation by metal cations toward nucleophilic addition (MeOH) conducted in conjunction with its characterization revealed that the relative reaction rates correspond beautifully to the established stabilities of the resulting metal complexes (8-hydroxyquinoline, $Cu^{2+} > Ni^{2+} > Zn^{2+} > Mn^{2+} > Mg^{2+}$).[29] This provides the opportunity to

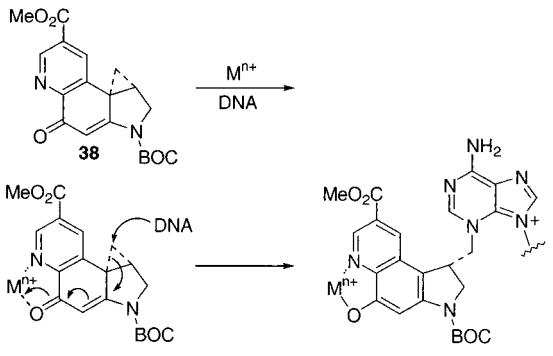

Figure 25.

predict, control, and tune the reactivity over a wide range depending on the application and conditions. Notably, Mg^{2+} provided a rate which was not distinguishable from a competitive background rate for methanolysis removal of the BOC group, indicating that this prominent endogenous metal cation, like Na^+, does not activate CPyI effectively.

Consistent with this behavior, the efficiency of the DNA alkylation reaction for **38**, but not **6** or **7**, was dramatically increased in the presence of $Cu(acac)_2$ (100×), $Ni(acac)_2$ (100−1000×), and $Zn(acac)_2$ (1000×).[28] This enhancement increased with increasing metal cation concentration for **38** and resulted in no change in the DNA alkylation selectivity. Under the conditions studied, the enhancement was especially remarkable with $Zn(acac)_2$ (1000×), which promoted DNA alkylation of **38** at concentrations as low as 10^{-5} M in **38**. Alkylation at such low concentrations is unprecedented for such simple alkylation subunits, and this efficiency is within 10-fold of the natural products (+)-duocarmycin SA and CC-1065 (10^{-6} M, 25 °C), Figure 1. Similar enhancements in the rates of DNA alkylation were also observed. For example, *N*-BOC-CPyI (10^{-2} M) provided complete consumption of w794 DNA within 10−20 min (25 °C) in the presence of 100 equiv of $Zn(acac)_2$ while only ∼10% reaction was observed after 24 h (25 °C) in the absence of the metal cation, providing a rate enhancement of DNA alkylation >1000×.

Intriguingly, comparative trace metal analysis of cancerous and noncancerous human tissues have revealed significant distinctions.[300,301] Although no generalizations were possible across all tumor types, there were significant and potentially exploitable differences within a given tumor type. For example, Zn was found in breast carcinoma at levels 700% higher than in normal cells of the same type, while lung carcinoma exhibited a reversed and even larger 10-fold difference.[300] Thus, chemotherapeutic agents capable of Zn activation might exhibit an enhanced activity against breast carcinoma attributable to this difference in Zn levels.

In addition to potential therapeutic uses, this class of agents has already been useful as a tool in studying the source of catalysis for CC-1065 and duocarmycin DNA alkylation reactions. Incorporation of CPyI into reversed and extended analogues of the natural products and ensuing mechanistic studies were recently described.[302] Taking advantage of the unique metal cation activation pathway of this class of agents, the effects of the DNA-binding domain (10^4-fold increase in DNA alkylation rate and efficiency) were quantitatively partitioned into two components: that derived from enhanced DNA-binding affinity and selectivity and that derived from a contribution to catalysis.

Consistent with the proposal that the DNA alkylation sequence selectivity originates in the noncovalent binding selectivity of the agents and not the properties of the alkylation subunit itself, reversed analogue **39** exhibited the remarkable reversal of the inherent enantiomeric DNA alkylation selectivity characteristic of the reversed analogues: (+)-indole$_2$-CPyI-BOC = *ent*-(−)-CPyI-indole$_2$, and *ent*-(−)-in-

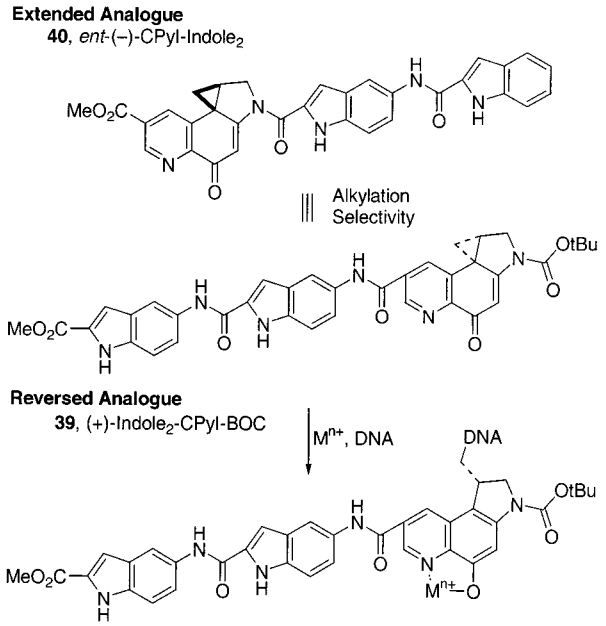

Figure 26.

dole$_2$-CPyI-BOC = (+)-CPyI-indole$_2$ (Figure 26). This conforms nicely to past observations and models (see above). It was also anticipated that **39** would be a poor DNA alkylating agent but that the addition and chelation of metal cations would promote an efficient DNA alkylation. This provided an additional opportunity to examine the impact of the DNA-binding domain on the rate and efficiency of DNA alkylation (**39** versus **40**), partitioning it into measurable contributions derived from minor-groove binding affinity versus activation.

The comparison of the rates of DNA alkylation by the reversed CPyI analogues, *N*-BOC-CPyI, and the typical extended analogue CPyI-indole$_2$ permitted the partitioning of the effects of DNA-binding subunits (21 000-fold) into that derived from increased binding affinity and selectivity (33-fold) and that derived from a contribution to catalysis (620-fold).[302] Thus, the rate difference observed between the reversed CPyI analogues and *N*-BOC-CPyI indicate that the DNA-binding subunits' binding affinity and selectivity increase the rate of DNA alkylation 33-fold. The comparison of CPyI-indole$_2$ and the reversed CPyI analogues indicates the DNA-binding subunits' larger contribution to catalysis is 250−5000-fold (620-fold for BOC derivative), a contribution that we suggest is due to a DNA-binding-induced conformational change that disrupts the stabilizing vinylogous amide conjugation. With the reversed CPyI analogues, addition of the metal cation Zn^{2+} artificially replaced this inaccessible source of catalysis, providing rates and efficiencies of DNA alkylation that were not distinguishable from those of the natural products or typical extended analogues without affecting the alkylation selectivity.[302] This complementary measure of the contributions of binding and catalysis to the alkylation rate available to CPyI analogues provided remarkably similar results.

These studies represented the first demonstration of the utility of metal-cation-induced in situ activation for use as a research tool as well as for the design of biologically active therapeutics. In this case, an exogenously added metal complexes with a DNA-bound agent, activating it for nucleophilic attack at the site of action. To our knowledge, this represents one of the first examples of a new mechanism of in situ activation that emerged from de novo design efforts versus those discovered in the course of understanding an existing compound's properties.

VIII. Conclusions

In situ activation of a number of clinically useful therapeutics targeting DNA has long been recognized to play an important role in the treatment of cancer.[119] Recent studies have led to the development of new agents utilizing familiar in situ activation reaction cascades and to the discovery of new in situ activation mechanisms. Many represent specialized subsets of prodrugs in which the activated or reactive species is generated near or at the site of action by endogenous or exogenous activation unrelated to the biological target. Endogenous activation may or may not contribute to the selective cytotoxic action of the molecules, while the latter fixes the site of action to the location at which the exogenous activation is provided. Of the endogenous activations, only reductive activation has been linked to the selective cytotoxic action of the molecules (e.g., mitomycin), where it is thought that hypoxic tumor cells permit higher intracellular concentrations of the reduced and activated agent. Of all the activation strategies recognized, only photochemical activation has been employed as an exogenous activation method for spacially defining the site of treatment accounting for the selective action. For the remainder, links between the activation method and the selective cytotoxic activity have not yet been made, and many may rely on other unrelated parameters (e.g., selective intracellular uptake). To date, those requiring activation by oxidation, reduction, nucleophilic attack, and elimination can all be regarded as examples of metabolic activation where the compounds themselves serve as a specialized set of prodrugs. Compounds requiring photochemical activation may be regarded as being uniquely dependent on exogenous activation, whereas the example of metal cation (Lewis acid) activation may be regarded as a rare in situ activation mechanism that does not involve a metabolic chemical reaction characteristic of a prodrug and that may be tuned to rely on endogenous or exogenous metal cation activation.

Distinct from these strategies is target-selective activation of a molecule upon reaching and binding its biological target. To our knowledge, the first well-defined example of this is the DNA-binding-induced activation of CC-1065 and the duocarmycins which results from a binding-induced conformational change in the molecule. This change disrupts the stabilizing vinylogous amide activating the molecule for nucleophilic attack only while bound to its target DNA. This may be viewed as related to mechanism-based suicide inhibitors of enzymes yet is distinct. Suicide inhibition relies on a targeted enzyme-catalyzed reaction to release a reactive, irreversible inhibitor within the enzyme active site, whereas the in situ activation of

CC-1065 and the duocarmycins does not. Although it also requires target activation, there is no intrinsic target-catalyzed reaction that is hijacked for the release of the reactive species and there is no chemical conversion to or release of a reactive species. One valuable future direction would entail linking such a target-selective activation mechanism to a tumor-specific molecular target.

As the structural and chemical features that contribute to successful examples of in situ activation become more fully understood, the de novo design of new in situ activation strategies and the extension of existing mechanisms to new classes of hybrid antitumor agents can be expected to emerge. These will find applications not only as the basis for new therapeutics, but also as basic research tools.

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