Chapter 1.

The Antitumor Antibiotic Mitomycins and FR Compounds

**The Anticancer Antibiotic Mitomycin Family**

The mitomycins are a well-known family of natural products first discovered in the 1950s, which exhibit antitumor and antibiotic activity. Mitomycin A and B (Figure 1-1, **1**,**2**) were isolated in 1956 by Hata and coworkers from the fermentation broth of *Streptomyces caespituous*.1 Two years later, Wakaki and coworkers isolated mitomycin C (**3**) from the fermentation broth of the same organism (Figure 1-1).2 The methylated analog of **3**,named poriformycin (**4**), was isolated from *Streptomyces verticillatus*.3 These four natural products represent the first known examples of the mitomycin family.

The structure of the mitomycins is quite unique among anticancer antibiotics. Structure elucidation studies of **1** using chemical degradation, UV spectroscopy,4 and X-ray crystallography5 showed the presence of an aziridine ring fused to a pyrrolo[1,2a]indole and aminobenzoquinone. Since **1** could be converted into **3** upon treatment with methanolic ammonia,4a structures **2**-**4** were assigned by analogy. The unique scaffold of **1**-**4** was later confirmed by Kishi’s total syntheses,6 and the absolute stereochemistry was revised in 1983 using X-ray dispersion techniques.7 These compounds were the first examples of aziridine containing natural products, and currently there are over 1000 synthetic analogs.8

The structurally unique fused aziridine pyrrolo[1,2-a]indole aminobenzoquinone core leads to a highly complex mechanism of action responsible for cellular toxicity. In order to

understand the relationship between structure, antitumor activity, and mechanism of action, the mitomycins have been the subject of intense study over the past half century.9



**Figure 1-1**. Mitomycins A, B, C and Poriformycin

During initial bioactivity studies, it was found that both **1** and **3** had good antitumor activity *in vivo* with dose ranges between 8-1000 µg/kg.1,2 However, the LD50 values for **1** and **3** in micewere found to be 2.0 mg/kg and 5.0 mg/kg, respectively; therefore, **3** is considered the “safer” cytotoxic agent. Ultimately, **3** became the best mitomycin candidate for clinical trials.10 It was found that hypoxic cancer cells (low O2 tension and a reducing environment) are particularly sensitive to **3** at low doses,11 while non-hypoxic cancer cells and non-cancerous cells also become sensitive to **3** at higher dose levels.12 Since these initial activity studies, the effective dose ranges and dosing schedule for **3** have been optimized, and mitomycin C has been shown to be clinically effective in treatment against a number of cancers.13 However, even at therapeutic doses, **3** can cause bone marrow suppression and gastrointestinal damage. Consequently, it is generally used as a last resort against cancers that do not respond well to more conventional treatments such as radiation. Therefore, Mitomycin C, marketed by BMS as Mutamycin®, has been a chemotherapeutic agent for the past forty years and is still used to treat breast, non-small cell lung, and head and neck cancers when used in combination therapy.14

An ultimate goal of the study of the mitomycins is the generation of synthetic analogs that have better selectivity for cancerous cells over non-cancerous cells but without the severe toxic side effects.9b,9c,11 Achieving this goal is unlikely without a thorough understanding of the mechanism of action of these structurally unique heterocycles. A multitude of mechanistic and metabolite characterization studies show that the mechanism of action is quite complex. Four main metabolites of **3** have been isolated upon exposure of DNA to **3** in cell cultures and under various conditions *in vitro*. The main metabolites isolated are the bis-alkylated DNA lesion **5**,15the monoalkylated DNA lesions **6**16and **7**,17and a reduced species 2,7-diaminoaziridinomitosene **8**18(Scheme 1-1). The formation of each metabolite is indicative of a different form of activation of the mitomycin. Therefore, the mechanism of activation leading to and the formation of each species will be discussed separately below.

**Scheme 1-1**. Main DNA adducts of **3**



**Events Leading to DNA Cross-linking by Mitomycin C**

Mitomycin C is a highly active anticancer and antibacterial agent due to its ability to form interstrand DNA cross-links. Although the reduction of the aminobenzoquinone of the mitomycins can lead to the generation of destructive super oxide radicals, hydrogen peroxide, and hydroxy radicals, which cause single strand breakage,19-21 the cytotoxic event leading to cellular death is actually the formation of interstrand DNA cross-links.22 Interstrand cross-links impede the replication fork and thereby halt replication of the DNA, ultimately resulting in cell death.23 These cross-links result in thermally stable double stranded DNA that “spontaneously” reanneals under denaturing conditions. However, mitomycin C does not react with purified DNA until it has been activated with a reducing enzyme or chemical reductants via a two-electron reduction or two one-electron reductions.22-25 Once the mitomycin is reduced, a mechanistic cascade initially proposed by Iyer and Szybalski is started that ultimately leads to a metabolite capable of cross-linking DNA (Scheme 1-2).24 Initial 2 electron reduction of **3** leads to the hydroquinone leucomitomycin **9**. Loss of the angular methoxy group followed by tautomerization to the indole forms the leucoaziridinomitosene **10** (so named due to loss of the methanol from C(9) and C(9a)).4a,24,26 In metabolite **10**,the two latent electrophilic sites of **3**, the aziridine and C(10) carbamate,26 are activated for heterolysis.

It has been shown that the C(1) position is the first reactive site27 and derivatives without the aziridine present do not retain the described DNA alkylating activity.28 Ring opening of the aziridine ring is facilitated by release of ring strain and stabilization of the resulting “benzylic” carbocation at C(1) in the quinone methide **11**.26 Once the alkylation at C(1) of **11** occurs, the C(10) carbamate is displaced in an SN1 manner by the indole nitrogen lone pair.29,30 The second alkylation event can then occur at C(10). Cross-linking of DNA occurs when an appropriately positioned deoxynucleoside of one DNA strand alkylates at C(1) followed by attack at C(10) by a deoxynucleoside on the complementary strand. Thus, the two electrophilic sites at C(1) and C(10) of **3** are essential for cross-linking activity.

The proposed active metabolite **10** was found to be extremely reactive and unstable.24 Due to the transient nature of the bis-alkylating metabolite, it cannot be isolated or characterized. However, various mechanistic investigations and characterization of the isolated DNA-**3** adducts provide evidence that supports the proposed mechanism of action. In efforts to elucidate the properties and structure of this bis-alkylating agent, the reactivity of reduced **3** in the presence of exogenous nucelophiles was investigated. Hornemann and coworkers found that reduction of **3** with Na2S2O4 in the presence of potassium ethylxanthate provided the bis-alkylated products **15b**, **16b** and monoalkylated products **15a** and **16a** (Scheme 1-3).31 The monoadduct was favored with fewer equivalents of the reducing agent, and it could be converted to the bis-adduct upon retreatment with Na2S2O4. This was the first chemical evidence that the reduced mitomycin could alkylate at both the C(1) and C(10) positions. Furthermore, the adducts were isolated in approximately a 1:1 *cis*:*trans* ratio suggesting that ring opening of the aziridine is an acid catalyzed SN1 process.

Bean and Kohn found that ethyl monothiocarbonate was also an effective nucleophile for C(1) and C(10) attack (Scheme 1-3, **15c**, **15d**, **16c**, **16d**).32 They could favor monoadduct formation to give **15c** and **16c** by lowering the temperature. Again, the isolation of both *cis* and *trans* isomers supports SN1 ring opening of the aziridine. Although these studies showed that C(1) and C(10) acted as electrophilic sites under reductive activation, the isolation and characterization of a cross-linked DNA adduct by Tomasz in the 1980s finally confirmed the mechanistic proposal made twenty years earlier.15a

**Scheme 1-2**. Reductive activation and alkylation of **3** by guanine nucleosides (dG)



**Scheme 1-3**. Mono- and Bis-alkylation of reduced **3** with exogenous nucleophiles



The cross-linked DNA lesion **17a** was obtained upon reduction of **3** with Na2S2O4 in the presence of *Micrococcus luteus* DNA.15a Analysis of the peracetylated lesion **17b** by 1H NMR spectroscopy, UV spectroscopy, and FTIR showed that C(1) and C(10) had been attacked by the exo amino (N2) moiety of two deoxyguanosines (Figure 1-2). Notably, Iyer and Szybalski reported in earlier studies that DNA with a higher percentage of CG base pairs had a greater occurrence of cross-link formation.24 Furthermore, three independent studies confirmed that cross-link formation is specific for the 5′CG3′ sequence.33–35 Using computer constructed molecular models and examination of the van der Waals contacts, Tomasz and coworkers observed a snug fit of the tetracyclic core of **3** into the minor groove of DNA resulting in only minor perturbation of the backbone.15a Surprisingly, Tomasz and coworkers found that reductively activated **3** can form intrastrand cross-links at the 5′GpG3′ sequence *in vitro*; however, there is a preference for interstrand cross-link formation (Figure 1-2).15b Thus, the long awaited isolation of the bis-alkylated DNA lesion of mitomycin C gave direct chemical proof of cross-link formation as proposed by Iyers and Szybalski.



**Figure 1-2**. DNA lesion of **3**, and schematic of interstand and intrastrand cross-linking.

Isolation of the DNA lesion cross-link confirms that reductively activated mitomycin C is a bis-alkylating agent at C(1) and C(10) capable of cross-linking complementary strands of DNA. However, the oxidation state of the bis-alkylating agent formed in the activation cascade of **3** has been the subject of much debate. Iyer, Szybalski, and others proposed that upon 2 electron reduction the nitrogen lone pair of the leucomitomycin facilitates expulsion of the angular methoxy group via iminium ion formation to start the activation process.22-26,36 However, 1H NMRstudies by Danishefsky showed that the leucomitomycin **19** formed upon hydrogenation of mitomycin **18** in pyridine does not spontaneously expel methanol to form the leucoaziridinomitosene **20** (Scheme 1-4).25,37 Furthermore, subsequent oxidation of **19** by air affords the parent mitomycin **18** without noticeable degradation.37 When the 1 electron reducing agent ascorbic acid was used for reduction of **18**, the quinone **23**,which had lost methanol, was isolated upon air oxidation.38 Initial formation of the semiquinone radical anion **21**,followed by loss of methanol and rearrangement would provide the semiquinone radical **22**. Finally, oxidation of **22** affords the observed product **23**.38  This mechanism for expulsion of the angular methoxy group at the quinone anion radical oxidation state was supported by Kohn’s observations that loss of methanol occurred upon 1 electron reduction of **3** in polar protic solvents under anaerobic conditions.39

**Scheme 1-4**. One electron reduction to initiate the activation cascade of *N*-methylmitomycin A

During the 1H NMR spectroscopy studies of the reductive activation of the mitomycins, Danishefsky and coworkers observed the elusive leucoaziridinomitosene **20**.40 Reduction of the aziridinomitosene **23** under hydrogenation conditions in pyridine provided the unstable **20**. Fortunately, **20** could be trapped as the isolable bis-triethyl silyl ether **24**. Notably, the successful generation of the putative bis-alkylating agent **20** allowed further mechanistic studies on the activation of the mitomcyins.

Since the semiquinone **21** was implicated in the expulsion of the C(9a) angular methoxy, and therefore, the start of the activation cascade, studies have tried to elucidate the possible role of the semiquinone in the alkylation events. Danishefsky found that the leucoaziridinomitosene **20** was not an efficient bis-alkylating agent when treated with potassium ethyl xanthate under aqueous pyridine conditions. However, reduction of **23** using catalytic Na2S2O4 did result in high yields of the alkylated products in aqueous pyridine.40 Crothers et. al. found that two-electron reduction of **18** in the presence of DNA,followed by 1 electron oxidation with FeCl3under anaerobic aqueous conditionsprovided a species that was a more efficient cross-linking agent than **20**.41 Importantly, the possibility that the hydroquinone is also a bis-alkylating agent was not ruled out. However, controlled electrochemical reduction to either the semiquinone or the hydroquinone of **3** in dimethylformamide (DMF) showed that the semiquinone was more stable than the hydroquinone25 consistent with Danishefsky’s observation of the instability of **20**. Thus, evidence has been provided that the leucoaziridinomitosenes at the hydroquinone oxidation state are too unstable and too inefficient as an electrophile to be the main bis-alkylating agent responsible for DNA cross-link formation.41

Although the hypothesis that the bis-alkylating agent is at the semiquinone oxidation state (**22**) is quite intriguing, it has not been unambiguously confirmed. Other analytical methods used during the reductive activation of the mitomycins do not substantiate claims of semiquinone radical formation during ring opening of the aziridine; however, they do not discount the formation of semiquinones such as **21** or **22** *in vivo*.42 Another puzzling observation by Hoey showed that dismutation of the semiquinone to the hydroquinone occurs before ring opening of the aziridine under aqueous buffer conditions.43 These inconsistencies may be due to the use of organic media instead of physiological conditions during Danishefsky’s and others work. As a result of the various observations, the debate about which oxidation state of the activated **3** is responsible for cross-linking is still ongoing.