Mitomycin C: small, fast and deadly (but very selective)

Mitomycin C, an important antitumor drug and antibiotic, has an extraordinary ability to crosslink DNA with high efficiency and absolute specificity for the sequence CpG. Recent results have shown how mitomycin C crosslinks DNA, and why the sequence specificity is so complete. This new understanding may allow the design of agents that mimic mitomycin C's economy of structure and can crosslink other sequences.

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Mitomycin C (MC; compound **1**, Fig. 1) is an antitumor antibiotic discovered in the 1950s by Japanese microbiologists in fermentation cultures of the microorganism *Streptomyces caespitosus* [1]. A number of close structural variants of MC have since been isolated, collectively called the mitomycins. MC has a variety of specific biological effects in mammalian cells, including selective inhibition of DNA synthesis, mutagenesis, and stimulation of genetic recombination, chromosome breakage and sister chromatid exchange, and induction of the DNA repair (SOS) response in bacteria [1]. It has therefore been widely used as a tool to study such phenomena. MC is also an important antitumor drug used widely in the clinic.

The molecular mechanism underlying the biological and pharmacological properties of MC is unprecedented. In 1963 it was shown that MC crosslinks the complementary strands of the DNA double helix; this is so lethal that a single crosslink per genome is sufficient to cause death of a bacterial cell [1]. No other natural antibiotic works this way, with the possible exception of carzinophillin, which has not been fully characterized structurally [2]. DNA crosslinking agents, typically bifunctional alkylators like MC, have been synthesized, however, and have been widely used in cancer chemotherapy. A complete understanding of MC's mechanism of action, the identity of the DNA adducts it forms and the structure of the DNA–MC complex would help us understand how MC induces cell-cycle arrest and apoptosis, and would be invaluable in the design of drugs that aspire to mimic MC's efficiency of action. New insight into these questions has come in recent years from high-resolution NMR studies and molecular modeling.

Reductive activation cascade of MC

MC itself (structure **1**, Fig. 1) does not react with DNA. Upon enzymatic or chemical reduction of the quinone, however, a cascade of spontaneous transformations ensues, culminating in the opening of the aziridine ring to produce the unstable vinylogous quinone methide **2**, which has high alkylating reactivity at the C1 position (Fig. 1; [1,3–7]). When compound **2** reacts with a DNA

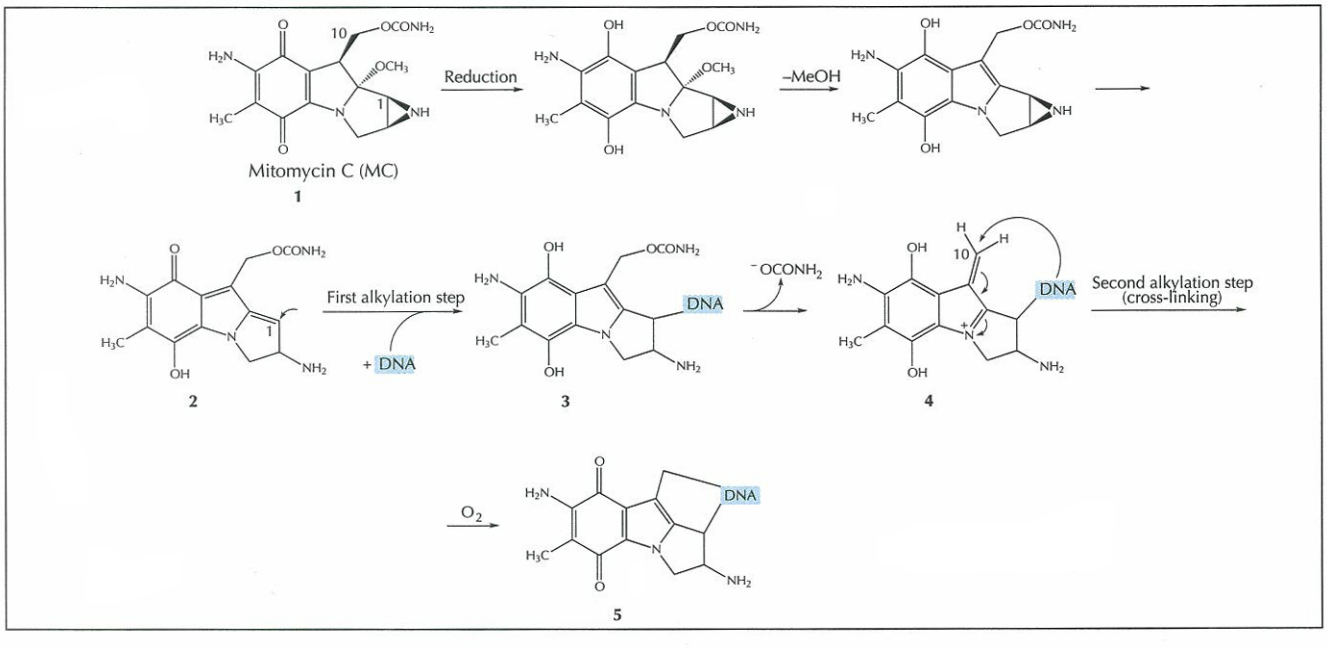

Fig. 1. The reductive activation cascade of mitomycin C (MC) [1,3–7]. Reduction of the quinone of MC initiates a series of spontaneous transformations culminating in the opening of the aziridine ring. The activated MC attacks DNA twice, resulting in covalent cross-linking of the strands of the DNA duplex.

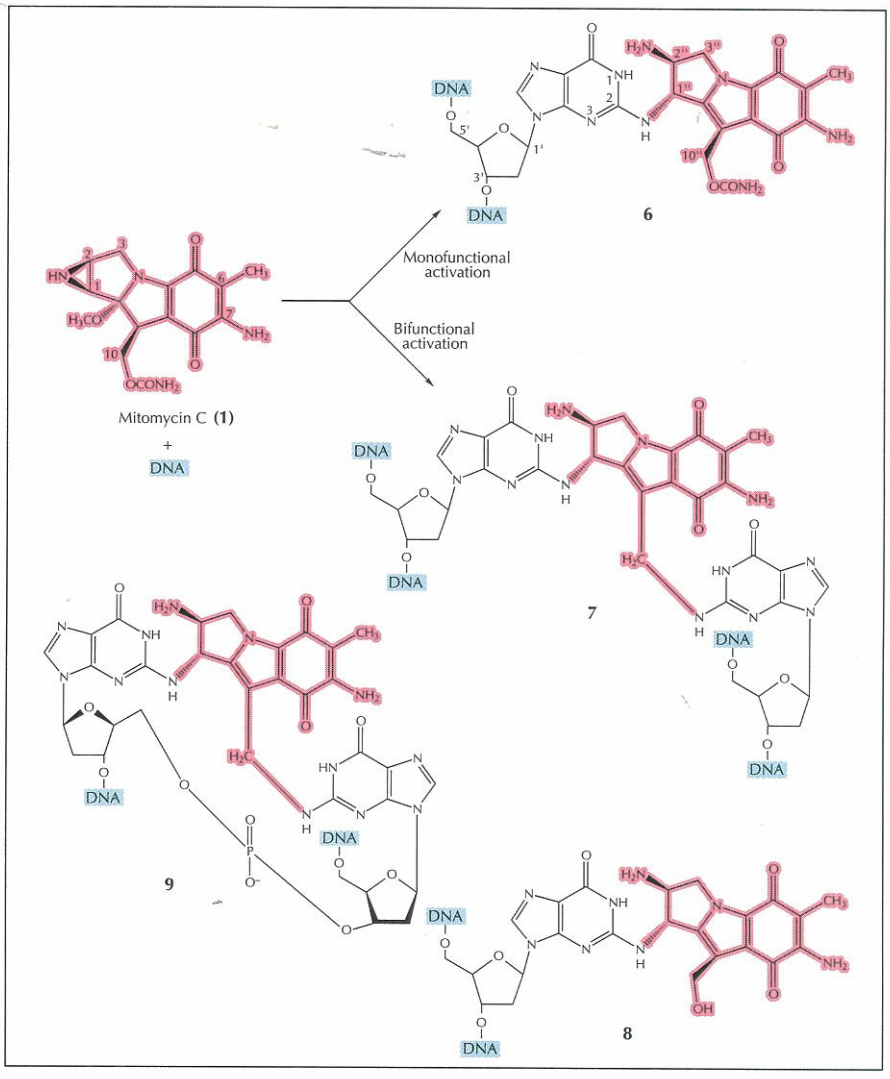

Fig. 2. Adducts of MC and DNA formed by reductive activation. Activation of only one of the alkylating functions gives monoadduct **6**, whereas activation of both alkylating functions can give products **7–9**. MC is highlighted in pink.

nucleophile at C1 (to form adduct **3**) a second alkylating center develops at C10 by reverse Michael elimination of the carbamate (adduct **4**); this adduct is attacked by another DNA nucleophile to give the reoxidized DNA crosslink (adduct **5**). Reduction thus converts MC into a highly reactive bifunctional alkylating agent. Several flavoreductases are known to catalyze this transformation in bacterial and mammalian cells [8].

It is worth noting, in passing, that this activation scheme is remarkably similar to the reductive mechanism that activates another group of antibiotics, the enediynes (which have an entirely different type of structure and damage DNA by cleaving rather than crosslinking it). The DNA-cleaving activity of the enediyne dynemicin A, for example, is triggered by reduction of a quinone, resulting in opening of an originally inactive epoxide ring assisted by quinone methide formation [9]; this mechanism is strikingly similar to the assisted ring-opening of the originally inactive aziridine of MC, leading to quinone methide **2**.

The fact that MC is activated by reduction is important in the selectivity of its antitumor activity. Many solid tumors are short of oxygen compared to normal tissues. As activation of MC is inhibited by an oxidizing environment, MC has selective toxicity for these hypoxic solid tumors, and potently suppresses their growth [8]. This feature of MC's design has frequently been imitated by pharmaceutical chemists; many new antitumor agents have been designed that are triggered by bioreduction, and several of them are under clinical development (see, for example, [10]).

Structures of MC–DNA adducts

The chemical identity of the DNA crosslinks induced by reduced MC has been determined by isolating crosslinked nucleosides from MC-treated DNA. Monofunctionally alkylated nucleosides (monoadducts), formed at the same time as the crosslinked adduct were also isolated. The structures of all the major MC–DNA adducts formed in cell-free systems are shown in Figure 2. Under reductive conditions, MC alkylates only the 2-amino groups of guanine nucleosides, in the minor groove of DNA. The first alkylation step by the quinone methide **2** leads to monoadduct **6** [11]; in some conditions, this is the only alkylation that occurs. Under conditions in which the second alkylating center develops,

however, bisadduct **7**, which forms a crosslink between the DNA strands, is a major product [7]; less frequently, the second alkylation step yields bisadduct **9**, a crosslink within one strand [12]. The 10"-decarbamoylated monoadduct **8** is also a major product of bifunctional activation, and results from nucleophilic attack by water at the C10" position [13]. The same adducts have been detected and identified *in vivo* [7,14].

Structure of MC–DNA complexes

Early molecular modeling studies indicated that, like most natural antibiotics targeted at DNA, the MC molecule resides in the minor groove in the monoadducts [11,15–17] and in the crosslinked adducts [7], and does not greatly perturb the structure of duplex DNA. A study in which the structure of a crosslinked hexanucleotide duplex was examined with two-dimensional NMR and restrained molecular mechanics computation was largely consistent with these models [18]. The structure of the monoadducts in DNA has become available only recently. A high-resolution structure of the MC monoadduct **6** formed with a nine-base-pair oligonucleotide duplex was obtained using two-dimensional NMR and restrained molecular dynamics calculations, including NOE-intensity-based refinement ([19]; Fig. 3). In this structure, the mitomycin residue is lying inside the slightly widened minor groove, resulting in extensive non-covalent contacts between the drug and the DNA duplex. The indoloquinone ring system is found to be stacked on one side of the duplex along the backbone of the non-bonding strand. Although the details are different, this structure makes it clear that the most important elements of the earlier models were correct. It also clarifies several aspects of MC's mechanism of action and sequence selectivity.

Both alkylation steps are sequence-specific

The first, monoalkylation step in the formation of the crosslinked adduct is selective for guanine nucleosides in the sequence 5'-CpG-3' (hereafter CpG) [20,21], while the second step is absolutely specific for the same sequence [22–24]. The selectivity of the first step is not dramatic; alkylation at CpG is on average five-fold [25] or ten-fold [21] enhanced over that at other NpG sequences. The second step, however, absolutely requires a G opposite the C. G residues flanking the NpG sequence on the opposite strand are never crosslinked, though intrastrand crosslinks can form on NpGpG sequences. The selectivity of the first step, though not absolute, is crucial for successful crosslinking and for the biological activity of MC, as we will see below.

Surprisingly, the selectivity of the monoalkylation step requires not only a CpG sequence on the target strand, but also a G residue on the opposite strand. The selectivity of MC for CpG can be abolished by replacing the guanine nucleoside in the opposite strand, which pairs with the C of the CpG, with inosine. Thus, the 2-NH_2 group of the opposite-strand guanine is essential for selective monoalkylation. It was proposed that this group

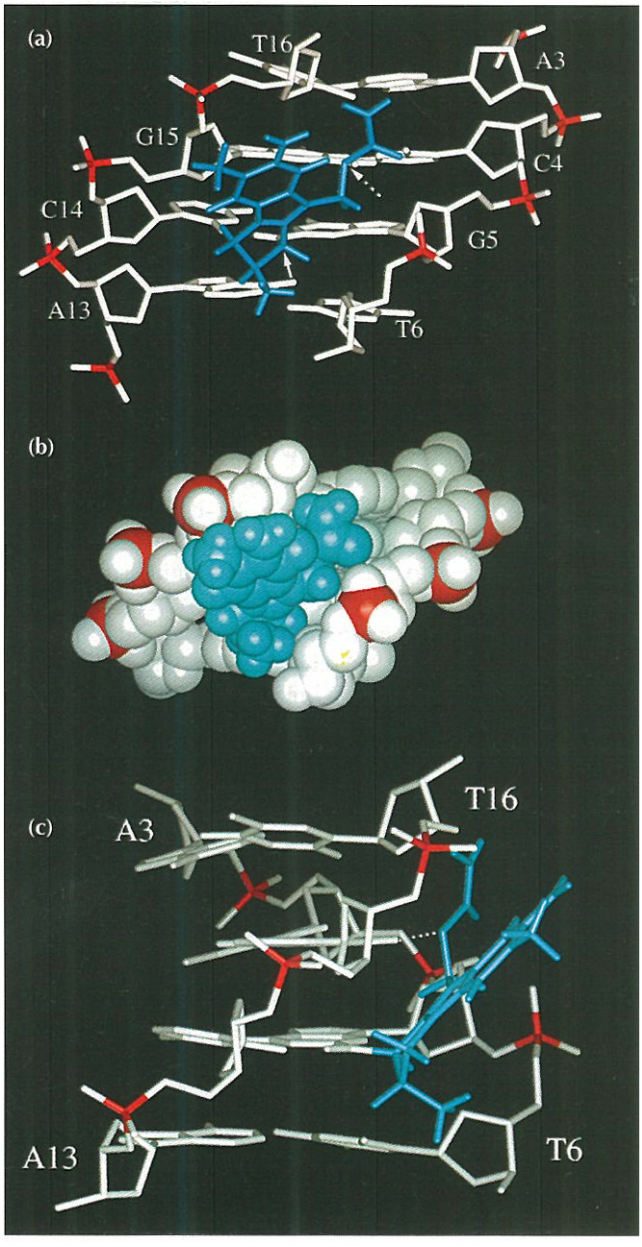

Fig. 3. Structure of the MC–DNA monoadduct. **(a)** Minor-groove view of the central four base pairs of the monoalkylated complex between MC and the double-stranded oligonucleotide d(I-C-A-C-[MC]G-T-C-I-T)•d(A-C-G-A-C-G-T-G-C). I, inosine; residues are numbered sequentially from the left. The MC residue is shown in blue. The white solid arrow indicates the covalent linkage of C1" of MC to G5 as in adduct **6**. The white dotted arrow points to the C10" oxygen atom of MC and the exposed N^2 hydrogen atom of G15, which are located close enough together to form an H-bond. **(b)** Same as (a), using space-filling model. The one-sided stacking of the MC chromophore with the backbone of the non-bonded strand (left side) is apparent. **(c)** Model in (a) is rotated to make visible the H-bond between the C10" oxygen atom of MC and the exposed N^2 hydrogen atom of G15 (white dotted line).

can form a specific H-bond with the C10" oxygen atom of activated MC (see Fig. 2) and that this non-covalent bond increases the rate of formation of the covalent bond with the target guanine [21,25] (Fig. 4). Modeling studies [11,15–17,25] supported this suggestion, and the recent

high-resolution structure of the MC–oligonucleotide monoadduct [19] decisively shows that the MC residue is indeed aligned in the minor groove to form this specific H-bond (Fig. 3). One can assume, therefore, that before the first covalent bond between MC and DNA is formed the opposite-strand G has already been recognized by H-bonding, and that the monoadduct structure is a trapped form of this pre-covalent complex.

It has been very difficult to examine the non-covalent binding of MC to DNA directly. Unreduced MC does not bind to DNA and the reductively activated form, compound **2**, is too short-lived for direct study. Nevertheless, there is evidence for the non-covalent binding of compound **2** to DNA from two different investigations [22,26]. Our laboratory [26] used the stable oxidized form of compound **2**, 2,7-diaminomitosene, which is positively charged, and found that it intercalates non-specifically into DNA, with a binding constant in the range of 10^4 M^{-1}. Although this does not help us to examine the CpG-specific minor-groove binding that precedes the covalent reaction, this bulk, non-specific binding mode is presumably important to attract activated MC to DNA. The CpG-specific minor-groove binding is an alternative pre-covalent binding mode, in equilibrium with non-specific binding. It is interesting to note that the unnatural enantiomer of MC recognizes CpG in DNA by the same mechanism as MC, as indicated by the CpG-specificity of the alkylation, the similar orientation of the compound in the minor groove and the identical chirality of the drug–DNA bond [27].

As noted above, crosslinking by MC is absolutely specific for the duplex DNA sequence CpG•CpG (Fig. 5); crosslinks to neighboring guanines never form. What determines the remarkable specificity of the second alkylation step? In the high-resolution NMR structure of the monoadduct–oligonucleotide complex (Fig. 3) it can be clearly seen that the monoadduct is oriented in the minor groove so that its C10" carbamate is pointing towards the

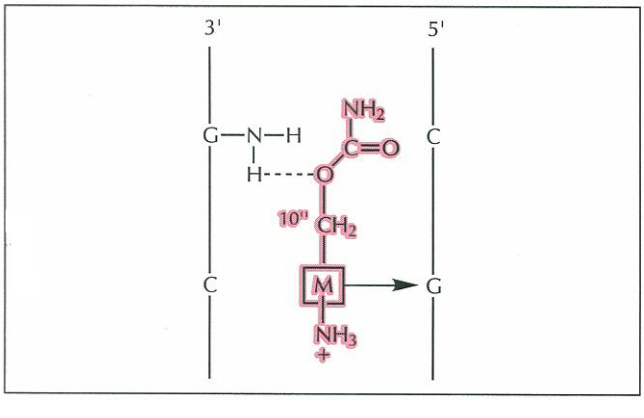

Fig. 4. Schematic illustration of a CpG sequence-specific H-bond (dotted line) between the C10'' oxygen atom of the activated form of MC and DNA. The structure in pink symbolizes activated MC (**3**); its 10''-carbamate and 2''-NH_3(+) functional groups are shown. The arrow points to the guanine nucleoside that is targeted for the covalent reaction.

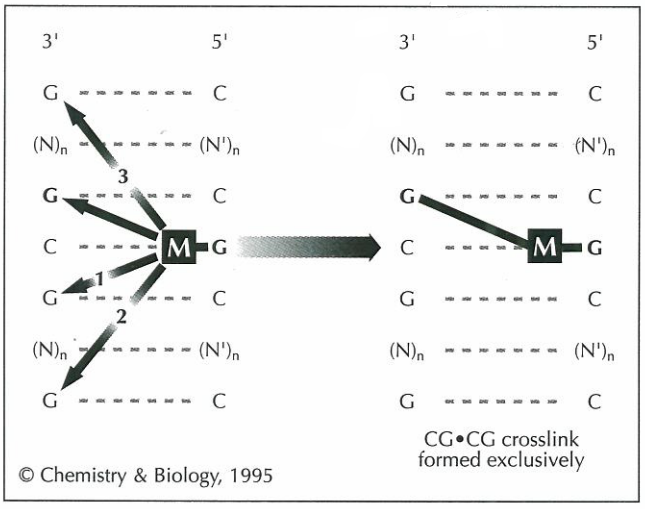

Fig. 5. Exclusive cross-linking of the CG•CG sequence by the MC-monoadduct. Arrows point to guanines in different positions relative to the monoadduct which are potential targets to form a crosslink. The solid arrow points to the only cross-link observed experimentally. Broken arrows 1–3 would lead to GC•GC, $GN_nC•GN'_nC$ and $CN_nG•CN'_nG$ crosslinks, respectively. Such cross-links are formed frequently with other cross-linking agents but not with MC.

5' end of the strand to which it is linked (therefore, towards the opposite-strand G), and is close enough (within 3.3 Å) to the N^2-atom of the unbonded guanine to form an H-bond with it. Thus the monoadduct is poised to form the crosslink without significant structural reorganization of the DNA [19]. At other sequences the monoadduct C10" position does not have a second N^2 atom within range, and is attacked by water to yield compound **8**. The covalent monoadduct is trapped by the C1" covalent bond to DNA in the proper orientation to form a crosslink; although the H-bond between the C10" oxygen atom and the N^2 hydrogen atom of the unbonded guanine is necessary to enhance formation of the monovalent adduct, once the covalent bond is formed, the H-bond is no longer essential to maintain this orientation [19].

Why be sequence-selective?

Reduced MC has strong intrinsic base- and regio-specific reactivity for the N^2 of guanine, even in a monomeric form [28]. Thus, MC requires two G residues on opposite strands to form a crosslink. The only way to be sure that there will be two G residues close to each other on opposite strands is to select for a CpG sequence. The pre-covalent sequence selectivity described above is thus a mechanism to ensure that MC monoalkylates only those guanines that will allow crosslinking. As a result, relatively few of the available drug molecules will be wasted by alkylating DNA at sites such as ApG, TpG and GpG, where only monoadducts (which are less often lethal) can be formed. The ability of MC to selectively recognize and alkylate its crosslinkable target must have made it a more potent weapon against competing microorganisms. Although other natural products seem to be sequence-selective for no good teleological reason, the

sequence selectivity of MC appears to be the result of natural selection to optimize its biological function.

Drug design: making a good machine even better

The site-specific, non-distorting, highly efficient crosslinking mechanism of the natural product MC stands in sharp contrast to the generally non-specific action of synthetic antitumor crosslinking agents, for example the N-mustard mechlorethamine, or the quinone-aziridines diaziquone (AZQ) and diaziridinyl benzoquinone (DZQ), which produce heterogenous mixtures of DNA crosslinks in low yield, accompanied by a large excess of non-specific monoadducts [29,30]. MC, in contrast, is an efficient DNA crosslinking machine. With the mechanism of this machine now well understood, it is not difficult to imagine new synthetic or semisynthetic structures that imitate MC, preselecting crosslinkable DNA sequences and using the same efficient crosslinking chemistry. It may be possible to design structures that crosslink the same sequence with improved efficiency, or that crosslink different sequences. Specificity for the sequence CpG, which is rare in mammalian genomes, does not seem ideal for a compound that is used as an antitumor agent. Only 5 % of all guanines in the mammalian genome are present in the CpG context, compared to 70 % (!) in the case of *Micrococcus luteus* DNA, for example. As a result, mammalian DNA is crosslinked by MC relatively poorly [1]. Although the sequence specificity of MC was presumably advantageous for its original purpose, improving the chances of killing competing microorganisms, it must be a disadvantage for killing tumor cells. Retaining the potent crosslinking mechanism of MC but altering or even relaxing its sequence specificity may allow the design of crosslinking agents that are more efficacious against tumors than MC itself.

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