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Review

Re-evaluation of the Molecular Pharmacology of Mitomycin C

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INTRODUCTION

Mitomycin C (MMC) is a naturally occurring antibiotic that was isolated in 1958 from the fermentation broth of *Streptomyces caespitosus*. It was shown to exhibit a broad spectrum of antitumour activity in preclinical animal screens and less toxicity compared to other mitomycins, and was introduced into clinical trials in Japan where anticancer activity was confirmed in humans [1]. MMC, which remains an important component in combination chemotherapy of breast, lung and prostate cancer, is among the few drugs to possess even marginal activity against colorectal cancer, and is probably the drug of choice for intravesical administration in superficial bladder cancer [2].

A number of recently published studies have extended and challenged many of our longstanding views on the molecular pharmacology of MMC, particularly in the areas of mechanism of action, DNA adduct profiles, enzymology of metabolic activation and drug metabolism. Therefore, it was considered timely to re-evaluate these areas and attempt to present a picture which is both cohesive and consistent.

MECHANISM OF ACTION

In 1964, it was first proposed that MMC required biotransformation, preferentially under anaerobic conditions, before drug activation occurred resulting in crosslinking of DNA [3]. To this day, this mechanism, termed anaerobic bioreductive alkylation [4], is generally accepted as holding true, even though considerably more details are now available on the type of metabolism, the chemical intermediates involved, the types of DNA adducts formed and the sequence specificity of alkylation.

The first stage in activation of MMC is quinone reduction,which can be catalysed by several different enzymes, either through a one electron pathway producing a semiquinone free radical intermediate or a two electron pathway producing a hydroquinone intermediate (Figure 1). In the presence of oxygen, the semiquinone will enter into a redox cycle, which although evolving reactive oxygen species, is generally accepted as not being critical for antitumour activity (pathway 2, Figure 1) [5, 6]. Generation of alkylating moieties by two electron reductases is unimpeded by molecular oxygen. After bioreduction, the C9a *O*-methyl group leaves the mitosane nucleus as methanol, a C9, 9a double bond forms producing the mitosene nucleus followed by proton assisted aziridine ring opening to generate an electrophilic carbon centre at C1 which alkylates DNA [4]. In this scheme, the key intermediate formed is a quinone methide (QM) [7]. Once the QM bonds to DNA, this promotes the carbamate group to leave yielding a C10 reactive centre which acts as the second point of attachment to produce DNA crosslinks [8, 9]. While MMC possesses no DNA binding activity, the QM is believed to bind non-covalently to DNA with high affinity by non-specific intercalation through a mechanism which is dependent on the ionisation of its 2-amino group and is enhanced under acidic conditions [10]. Reversible non-covalent binding can be viewed as a precursor event to irreversible covalent bonding. The QM reaction scheme is the pathway of MMC metabolic activation which is accepted by the majority of workers but at least three different alternative reaction schemes have been proposed [11–13].

It has been demonstrated recently by purely chemical means that, rather than acting as an electrophile at neutral pH (resulting in DNA bonding) (pathway 3, Figure 1) and a nucleophile at acidic pH as originally hypothesised (pathway 1, Figure 1) [7], the QM is predominantly nucleophilic in character at all functional pHs between 5.5 and 8.5 [14]. This theory does not challenge the concept of the QM, but explains why 2,7-diaminomitosene (2,7-DM, the product of the QM acting as a nucleophile) is the major metabolite of MMC seen *in vivo* (see below). It also suggests that DNA bonding is precluded unless the QM is in close proximity to a nucleophilic centre on DNA, otherwise it will preferentially react with a solvent proton to yield 2,7-DM. The electrophilic metabolites of MMC—1,2 *cis* and 1,2 *trans* 1-hydroxy 2,7-diaminomitosene (*cis* and *trans*-hydro)—are now proposed to be derived from a 7-aminoaziridinomitosene intermediate. This, in turn, is the oxidised form of the MMC quinone-reduced intact aziridino product, leucoaziridinomitosene (LAZM) (Figure 1) [14]. Since 7-aminoaziridinomitosene is only likely to form under more alkaline conditions, this explains why the *cis*- and *trans*-hydro metabolites are also only observed at more alkaline pH [11,14].

Based on an extensive series of biological studies employing the enzyme DT-diaphorase to activate MMC, two other key

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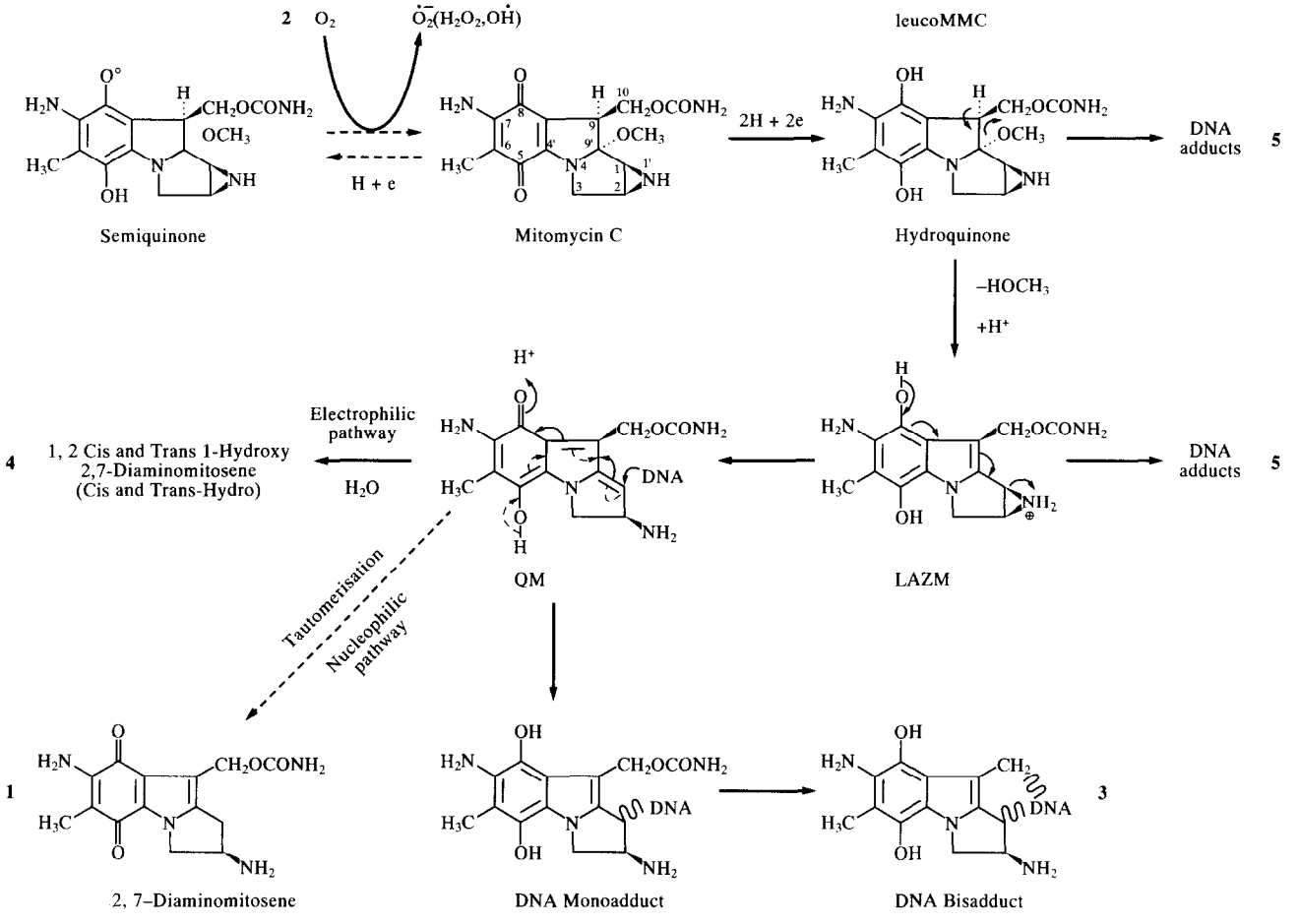

Figure 1. Proposed pathways of metabolic activation of mitomycin C (MMC) after one or two electron quinone reduction. Pathways are explained in more detail throughout the text.

intermediates have been proposed together with the QM as the precursors to DNA covalent bonding (pathway 5, Figure 1) [15]. These are the quinone-reduced intact aziridino products, leucoMMC (fully reduced MMC) and LAZM (fully reduced MMC minus the C9a *O*-methyl group), and their dependence on proton-assisted aziridine ring opening to yield electrophilic intermediates has been cited to explain the pH dependence of MMC DNA interstrand crosslinks (ISC).

COVALENT MODIFICATION OF DNA

A variety of MMC DNA adducts have been reported. It has been claimed that the major sites of covalent modification are at *O*-6 guanine followed by *N*-6 adenine and then *N*-2 guanine [16]. In a series of elegant studies, Tomasz's group has demonstrated that *N*-2 guanine is the preferred site of monoalkylation (90% of all DNA bonding) after bioreduction and chemical reduction, and identified the first bifunctional alkylation product as the *N*-2 guanine/*N*-2 guanine crosslinked adduct [9, 17]. The second site of attachment on MMC was the C-10 position, as originally hypothesised by Iyer and Szybalszi in 1964. Computer models have indicated that this bisadduct fits snuggly into the minor groove of DNA with minimal distortion to the structure of B-DNA, and have provided a rationale for the base specificity of MMC alkylation for guanine [17]. Using molecular biology techniques, an absolute requirement for guanine has been confirmed, and a sequence selectivity of ISC for 5′-CpG repeats has been demonstrated [18]. Studies by several independent laboratories have confirmed *N*-2 guanine as the principal site of covalent attachment to DNA [19, 20]. As well as ISC, MMC forms a dGpdG intrastrand crosslinked adduct [21]. An orientation model has been proposed to explain the sequence selectivity of MMC crosslinks [22, 23]. The C-1 mononfunctional adduct complexed at *N*-2 guanine (which always forms first) can only point in one direction where the C-10 carbamate group of MMC is facing the 3′ direction of the minor groove. Therefore, only when a G is situated 3′ of the monofunctional adduct in the complementary strand of DNA can a crosslink possibly form. This case is only satisfied with CG.CG or less frequently with GG.GG.

After metabolic activation with DT-diaphorase, an *N*-7 guanine adduct appears to account for greater than 90% of all MMC covalently bonded to DNA [24], and this probably reflects the unique manner in which the enzyme metabolises MMC. At pH 7.8, where predominately electrophilic intermediates are evolved, enzyme inhibition ensues as a result of protein alkylation and crosslinking [25]. At more acidic pH (5.8), nucleophilic intermediates are evolved resulting in the efficient formation of 2,7-DM. This metabolite can then act as a substrate for further bioreduction by DT-diaphorase, yielding the *N*-7 guanine monofunctional adduct complexed to C-10 of MMC. Evidence in favour of this proposal was presented with the finding that, when 2,7-DM was used as a substrate for DT-diaphorase instead of MMC, similar patterns of DNA alkylations to MMC were observed. Thus, DT-diaphorase-mediated DNA

monoalkylation by MMC may have an absolute requirement for 2,7-DM metabolite formation. Other enzymes involved in MMC metabolic activation, such as cytochrome P450 reductase, xanthine oxidase and xanthine dehydrogenase, are not inhibited at physiological pH, although their activity can fall significantly from pH 6.0 to 7.4 [26, 27]. The mechanism of DT-diaphorase inhibition has been speculated to be due to the orientation of MMC in the active site of the enzyme, bringing it in close proximity to specific nucleophilic sites on the protein [15]. *N*-2 guanine alkylation and minor groove 5'-CG ISC can also be detected after DT-diaphorase-mediated metabolism, but these were believed to be derived from the leucoMMC and LAZM intermediate forms of MMC and were only minor products [24].

The chemical structure of all the major *in vivo* DNA adducts formed in EMT6 mouse mammary tumour cells after exposure to MMC has recently been reported [28]. The major species detected under aerobic conditions were the *N*-2 guanine–*N*-2 guanine crosslinked adduct, followed by the decarbamoyl *N*-2 guanine monofunctional adduct, then the dGpdG intrastrand crosslink and finally the *N*-2 guanine monoadduct. These four adducts correspond to the same species that are formed *in vitro* after either chemical reduction or enzyme catalysed metabolism [9, 17, 21, 29]. A fifth dG adduct, codenamed Y, was detected but not identified chemically, and this product may represent the *N*-7 guanine adduct produced by DT-diaphorase. Under hypoxic conditions, levels of crosslinked adducts increased significantly, along with adduct Y. Treatment with the DT-diaphorase inhibitor, dicoumarol (DIC), resulted in elimination of the *N*-2 monofunctional adduct and a significant reduction in Y under hypoxic conditions, but produced large increases in both the inter- and intrastrand crosslinks. These results add weight to the view that the DNA modifications induced by DT-diaphorase are principally monoalkylations, and suggest that bifunctional alkylations are generated predominately under hypoxia by one electron reductases, such as cytochrome P450 reductase, cytochrome b5 reductase and xanthine oxidase, all of which have been shown to be present in this cell line [30–32]. This conclusion, if true, has major significance since it is generally recognised that crosslinks are more lethal to cells than monofunctional adducts [28, 33, 34]. In aerobic conditions, DIC still eliminated the *N*-2 monofunctional adduct, reduced Y but also reduced the number of crosslinks. Interestingly, DIC enhanced the formation of a sixth DNA adduct, codenamed X, particularly under hypoxia, and this has been attributed to the action of xanthine dehydrogenase, which has been previously isolated from EMT6 cells and shown to be stimulated 6–7-fold by this agent [31].

ENZYMOLOGY OF METABOLIC ACTIVATION

Several enzymes can catalyse the *in vitro* metabolic activation of MMC, and these include cytochrome P450 reductase [26, 29], xanthine oxidase [26, 29] and cytochrome b5 reductase [30] acting as one electron reductases; and DT-diaphorase [20] and xanthine dehydrogenase [31] acting as two electron reductases. Cytochromes P450 are believed not to transfer electrons directly to MMC, but may facilitate quinone reduction at least 2-fold through drug binding [32]. This view has been recently challenged where cytochromes P450 have been shown to reduce MMC to a semiquinone free radical, which caused toxicity to hepatocytes by alkylating reduced glutathione [35]. The hierarchy of participation from each of these reductases to metabolic activation of MMC *in vivo* has still to be established, but it is emerging that under different physiological conditions different enzymes may prevail [36]. Cytochrome P450 reductase is believed to predominate in artificially hypoxic cells [37, 38], although there have been reports that reduced expression of this enzyme leads to resistance only in normo-oxic cells [39]. Numerous studies with cancer cell lines support a major role for DT-diaphorase in MMC metabolic activation in normally oxygenated cancer cells but not in hypoxic cells [40–44]. However, a recent study has shown that DT-diaphorase can reduce MMC with equal facility under aerobic and anaerobic conditions in HT-29 human colon cancer cells [45].

A new role has been suggested for DT-diaphorase in the metabolic activation of MMC and its closely related analogue indoloquinone, EO9 [46, 47], namely, that DT-diaphorase protects cells from the hypoxic cytotoxicity of these drugs. According to this hypothesis, cell lines which express high levels of DT-diaphorase are more resistant to MMC than cell lines which express low levels of the enzyme, but only under hypoxic conditions. Under normal oxidative conditions, the converse applied where a direct correlation was observed between DT-diaphorase expression and cytotoxicity, confirming many previous studies which have also shown this to be the case [40–44]. A major cornerstone in the hypothesis was the finding that DIC increased hypoxic cytotoxicity of MMC and EO9 preferentially in cell lines rich in DT-diaphorase (5–10-fold increase versus a 1–3-fold). However, results generated using extremely high concentrations (200 μM) of DIC should be viewed with great caution since this agent can also inhibit cytochrome b5 reductase [15] and cytochrome P450 reductase [48], stimulate xanthine dehydrogenase [26] and induce the formation of unique DNA covalent adducts [28].

While it is unclear what the biochemical basis is behind these results, similar observations have been made by other groups in different cell lines [28, 32, 45], indicating that this is probably a real phenomenon. Two valid propositions have been put forward to explain their results: (i) that one electron reductases are more effective at metabolising MMC than DT-diaphorase (but that DT-diaphorase prevails under hypoxic conditions); and (ii) that the semiquinone form of MMC (the product of one electron reduction) is more toxic than the hydroquinone form of MMC (the product of DT-diaphorase metabolism) under anaerobic conditions [46]. The second explanation can be discounted more easily since, under hypoxic conditions, due to rapid disproportionation of the semiquinone free radical, the hydroquinone form of MMC is believed to act as a common intermediate after both one and two electron reduction [11, 15]. The first explanation is probably more valid since it can be supported by an emerging body of results which indicate that DT-diaphorase is less effective than one electron reductases in MMC metabolic activation. Firstly, pH dependent alkylation of DT-diaphorase results in enzyme inhibition at physiological pH, but this effect is less pronounced with one electron reductases. Secondly, DT-diaphorase produces predominately monofunctional *N*-7 guanine DNA adducts which are believed to be less cytotoxic than the bifunctional crosslinks formed by one electron reductases [28]. Thirdly, all the major enzymes involved in MMC bioreduction exhibit very low affinity constants (K_m) for the drug, thus, neither one should show a preference for MMC as a substrate. Xanthine oxidase and cytochrome P450 reductase have a K_m of 2 mM [26, 49], xanthine dehydrogenase a K_m of 299 μM [27] whilst DT-diaphorase also has a low K_m[50]. Therefore, the enzyme which is present in largest amount is likely to carry out the majority of MMC bioreduction regardless of intrinsic catalytic activity, and the pattern of metabolic

activation produced will parallel the biochemical properties of that particular enzyme.

Recent results from our laboratory lend support to the above conclusion [51]. When MMC was incubated with whole homogenates of the mouse colon adenocarcinoma MAC 16, which is rich in DT-diaphorase, the pattern of metabolism recorded was characteristic of DT-diaphorase, that is no increase in activity under hypoxic conditions and inhibition by DIC at a lower concentration (10 μM). However, when the tumour was subjected to subcellular fractionation, three fractions were isolated with the ability to convert MMC into 2,7-DM (mitochondria, microsomes and cytoplasm) and the fraction containing DT-diaphorase exhibited the lowest intrinsic activity. In contrast, the MAC 26 tumour, which is low in DT-diaphorase, but contained the same highly active mitochondrial enzyme, produced a pattern of metabolism consistent with one electron reduction: stimulation of metabolism by hypoxia and higher levels of activity under hypoxia compared to MAC 16 [51].

Many human tumours, such as liver, colon, breast and non small cell lung cancer, are rich in DT-diaphorase, making this enzyme a good candidate for the enzyme-directed approach to cancer chemotherapy [52–54]. However, recent data with DT-diaphorase and MMC suggest caution when applying that approach to this drug and related compounds, such as EO9. In tumours that are high in DT-diaphorase, induction of hypoxia will only increase antitumour activity by a small margin, if at all, but this should be from a level which is much higher than in tumours low in DT-diaphorase. Tumours that are low in DT-diaphorase may be less responsive, but could become substantially more sensitive by the induction of hypoxia, if such modulation was achievable *in vivo*. Thus, on balance, tumours high in DT-diaphorase would appear to be optimal for treatment with MMC. The one caveat to this conclusion is that it is based on data generated *in vitro* with cell lines, and these studies do not take into account the obvious biological and physiological differences that exist in solid tumours and their cellular heterogeneity. With this in mind, it has been demonstrated in a panel of four human xenografts that an inverse relationship exits *in vivo* between DT-diaphorase expression and antitumour activity, and that DIC increased antitumour activity to a greater extent in the tumours expressing the highest levels of DT-diaphorase [53]. These data parallel more closely the *in vitro* scenario of hypoxic cancer cells treated with MMC [46, 47].

DRUG METABOLISM

Despite overwhelming evidence for the major role of drug metabolism in the mechanism of action of MMC and in its clearance from the body, no previously published preclinical (or clinical) pharmacokinetic study has reported detection of MMC metabolites in either plasma or tissues [55–58]. Recently, analytical techniques have been published which can detect *in vivo* MMC and its three primary bioreductive metabolites 2,7-DM, *cis*- and *trans*-hydro in rodent transplantable tumour tissue (the Sp 107 rat mammary carcinoma and MAC 16 and 26 murine colon cancer) [59]. Five MMC metabolites were detected in Sp 107 10 min after drug administration, including the three primary metabolites. These techniques have been applied to characterise further MMC metabolism *in vivo* in tumour tissue [36, 60]. In an initial study, metabolism was investigated in the MAC 16 tumour which is rich in DT-diaphorase. One single metabolite was detected, 2,7-DM, which was generated in a burst of activity that extended over 30 min after drug instillation [36]. The rapid clearance of 2,7-DM may reflect the fact that this metabolite is only an intermediate in a complex chain of metabolic events [8, 11]. *In vitro*, 2,7-DM only has a half life of 13 min when incubated with either cytochrome P450 reductase of xanthine oxidase [26]. Induction of hypoxia by administration of albumin microspheres resulted in a reduction in 2,7-DM formation, confirming that DT-diaphorase protects MMC from metabolic activation under anaerobic conditions [36]. In a separate study, 2,7-DM was shown to have a half life of 30 min after intratumoural (i.t.) injection of 1 mg MMC in the Sp 107 tumour and 5 min after i.t. injection of 100 μg. In that study, decarbamoyl 2,7-DM (DC 2,7-DM, a secondary metabolite) was also detected [60]. This metabolite may reflect bifunctional metabolic activation of MMC at C1 and C10, whereas 2,7-DM may reflect monofunctional metabolic activation [16, 17]. Levels of DC 2,7-DM were 5–10-fold lower than 2,7-DM. The formation of DC 2,7-DM was also much slower that 2,7-DM and its half life was 130 min. These *in vivo* data parallel closely the kinetics of DNA covalent bonding after treatment of cells with MMC, or incubation of the drug with purified cytochrome P450 reductase and xanthine oxidase in a cell free system [49, 61]. DNA bonding occurred in a rapid burst of activity during the first 6 min of drug incubation: by 20 min the levels of monofunctional DNA adducts appeared to plateau and thereafter there was a steady rise in the formation of crosslinks for a further 70 min [49]. The rapid appearance and disappearance of the major metabolite (2,7-DM) may explain why all previous preclinical pharmacokinetic studies have failed to detect this product in either plasma or tissues.

In those *in vivo* studies, tumour levels of 2,7-DM correlated better than MMC to antitumour activity [36]. *In vitro* studies have also identified a correlation between 2,7-DM levels and cytotoxicity in human colon cancer cell lines [40]. Together, these data may shed light on the nature of the ultimate cytotoxic species after MMC treatment in view of the fact that generation of 2,7-DM appears to be an obligatory step in *N*-7 monoalkylation of guanine [24].

CONCLUSIONS

The molecular pharmacology of MMC remains a very complex subject. Over the past five years, a number of seminal papers have expanded our understanding, clarified certain areas of controversy and at the same time challenged some of our longstanding opinions. The most significant development has been in defining the role of the obligate two electron reductase DT-diaphorase. While it was originally believed by some that this enzyme did not accept MMC as a substrate, it has now emerged that it occupies a unique and central role in the molecular pharmacology of the drug. Since this enzyme is expressed at very high levels in a number of human tumours, this role clearly has clinical significance. If DT-diaphorase is present at high levels, it will act as the main MMC metabolising enzyme, resulting in metabolic activation both under aerobic and anaerobic conditions. However, because the enzyme is not as efficient as one electron reductases in activating the drug, the enzyme effectively protects cells from the maximum degree of drug-induced damage possible under hypoxic conditions. When DT-diaphorase is present at lower levels, it may still predominate in aerobic cells because one electron reductases are effectively inhibited by oxygen. Under anaerobic conditions, one electron reductases will participate significantly and the drug may have greatly increased activity. Ultimately, the pattern of MMC metabolic activation, and hence its antitumour activity, will

depend on the balance of enzyme activities present in a tumour and on its oxidative state.

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