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COMMENTARY

Enzymology of Mitomycin C Metabolic Activation in Tumour Tissue

IMPLICATIONS FOR ENZYME-DIRECTED BIOREDUCTIVE DRUG DEVELOPMENT

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ABSTRACT. Mitomycin C (MMC) is the prototype bioreductive DNA alkylating agent. To exploit its unique properties and maximize patient responses, different therapeutic approaches have been investigated. Recently, the focus has concentrated on monitoring the levels of the proteins metabolizing the drug and relating these to activity in a regimen referred to as enzyme-directed bioreductive drug development. To be successful, it is important to understand the enzymology of metabolic activation not only in cell lines but also in solid tumour models. A general mechanism of action for MMC has now emerged that is activated regardless of the source of reducing equivalents, comprising three competing pathways that give rise to unique reactive intermediates and different DNA adducts. Partitioning into the pathways is dictated by chemical considerations such as pH and drug concentration. DT-diaphorase stands out in this mechanism, since it is much less effective at metabolizing MMC at neutral pH. At least five different enzymes can catalyse MMC bioreduction *in vitro*, and as many activities may be present in solid tumours, including a series of novel mitochondrial reductases such as a cytochrome P450 reductase. Competition between reductases for MMC appears to be based solely on protein levels rather than enzyme kinetics. Consequentially, DT-diaphorase can occupy a central role in MMC metabolic activation since it is often highly overexpressed in cancer cells. Although a good correlation has been observed in cell lines between DT-diaphorase expression and aerobic cytotoxicity, this does not hold consistently *in vivo* for any single bioreductive enzyme, suggesting revision of the enzyme-directed hypothesis as originally formulated. BIOCHEM PHARMACOL **56**;4:405–414, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. mitomycin C; *in vivo* enzymology; tumour tissue; DT-diaphorase; enzyme-directed bioreductive drug development; hypoxia

BACKGROUND TO HYPOXIC CELL-SELECTIVE BIOREDUCTIVE DRUGS AND ENZYME-DIRECTED BIOREDUCTIVE DRUG DEVELOPMENT

MMC‖ is an important clinically active drug commonly used in combination chemotherapy regimens to treat breast, lung, and prostate cancer and is probably the drug of choice for intravesical administration in superficial bladder cancer [1]. As the prototype member of a class of drugs referred to as bioreductive agents [2], elucidation of the fine detail of its mechanism of action continues to generate considerable interest and controversy [3, 4]. Bioreductive drugs require enzymatic activation prior to the generation of alkylating moieties [5]. In the case of MMC, this process is believed to be enhanced under reducing conditions and inhibited by molecular oxygen [6, 7]. Thus, it was originally the belief that MMC may possess preferential activity against difficult-to-treat solid tumours containing large populations of hypoxic cells known to be resistant to ionizing radiation and many conventional anti-neoplastic agents [7, 8]. Although MMC is capable of killing hypoxic cells *in vivo* [9], the majority of the evidence suggests that it actually exhibits very little selectivity for hypoxic cells [10, 11].

More recently, the focus has shifted to the enzymology of metabolic activation, and efforts have concentrated on measurements of tumour contents of bioreductive enzymes [4, 12]. With the observation that key bioreductive enzymes such as DT-diaphorase (EC 1.6.99.2; NAD(P)H:quinone oxidoreductase, NQO1) can be overexpressed in chemoresistant human tumours such as NSCLC and colon cancer [13–16], optimism exists that these enzymes may be targeted by bioreductive drugs in a more selective treatment of cancer [17]. This approach has become known as enzyme-directed rational drug design, and its successful application depends on three critical elements [12]:

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‖ *Abbreviations:* CHO cells, Chinese hamster ovary cells; DIC, dicoumarol; 2,7-DM, 2,7-diaminomitosene; MMC, mitomycin C; NQO1, DT-diaphorase; NSCLC, non-small cell lung cancer; QM, quinone methide; and ROS, reactive oxygen species.

1. Understanding the enzymology of bioreductive metabolic activation of the candidate drug and establishing a predominant role for a single bioreductive enzyme.
2. Profiling bioreductive enzymes (activity and expression) in human tumour biopsy specimens and establishing overexpression of the candidate enzyme in relation to normal tissues.
3. Establishing that a correlation exsits (*in vitro*, *in vivo*, and ultimately in patients) between the level of enzyme activity and chemoresponsiveness.

While considerable effort has been expended in attempting to validate this approach with MMC and related compounds [12, 18–20], these workers have confined their investigations largely to *in vitro* models [21]. In addition, several recent papers have emerged that have challenged many preconceived ideas concerning the molecular pharmacology of MMC [reviewed in Ref. 3], and it is only now beginning to become clear how these studies may impact on the enzyme-directed approach to new anticancer drug development. The aim of this commentary is, first, to review recent developments in MMC metabolic activation and enzymology. Second, it is our intention to broaden the arena to the subject of *in vivo* enzymology of MMC bioreduction. Finally, the commentary will conclude by discussing the ramifications of recent data for enzyme-directed anticancer drug development.

METABOLIC ACTIVATION OF MMC AND COVALENT MODIFICATION OF DNA

The first stage in metabolic activation of MMC is quinone bioreduction [6], which can be catalysed by several different enzymes (see below), either through a one-electron pathway producing a semiquinone free radical intermediate or a two-electron pathway producing a hydroquinone intermediate. In the presence of oxygen, the semiquinone will enter into a redox cycle [22], which, although producing ROS, is generally accepted as contributing only in a minor way to antitumour activity [23, 24]. Generation of alkylating moieties by two-electron reductases is unimpeded by molecular oxygen [25]. Under hypoxic conditions, the semiquinone free radical chemically rearranges into the more stable hydroquinone, which probably serves as a common intermediate in both one- and two-electron reduction [25]. After bioreduction, the C9a O-methyl group leaves the mitosane nucleus as methanol, and a C9, 9a double-bond forms, producing the mitosene nucleus (**11**, Fig. 1) [5]. At this point, a number of different reaction schemes leading ultimately to DNA covalent modification have been hypothesized. Only recently has it been clarified which intermediates are formed under what experimental conditions [26]. Initially, a QM intermediate (**15**, Fig. 1), exhibiting both nucleophilic and electrophilic properties, was postulated [27, 28] to explain the simultaneous production of both DNA adducts and several classes of stable metabolites [29]. However, other workers believe that formation of MMC DNA interstrand cross-links requires proton-assisted aziridine ring opening of a leuco-aziridinomitosene intermediate (**11**) [30, 31]. Major groove DNA mono-adducts are ascribed to further quinone reduction of the major metabolite of MMC 2,7-DM via a QM intermediate (**6**) [31]. Yet other workers have shown the QM to be predominately nucleophilic in character at all functional pH levels between 5.5 and 8.5, implying that it is unlikely to be involved in alkylation reactions with guanine [32]. Here, basic conditions are necessary to produce 7-amino-aziridinomitosene (**12**), a novel MMC reactive intermediate with the electrophilic characteristics capable of DNA alkylation. While none of the above mechanisms represents the whole picture, each plays a part in a global mechanism of bioreductive activation that displays considerable complexity (see Fig. 1) [26].

In this mechanism, leuco-aziridinomitosene (**11**) acts as the central branch point for competing pathways of monofunctional activation (path 1, Fig. 1) and bifunctional activation (path 2, Fig. 1). Through an autocatalytic cycle, **11** transfers electrons to intact MMC producing 7-amino-aziridinomitosene (**12**, path 1), which can then act as a C-1 monofunctional alkylating agent yielding two stereoisomer N2 guanine minor groove DNA adducts [33, 34]. Path 1 predominates at neutral pH, and under these conditions monofunctional DNA adducts can account for up to 90% of all MMC DNA modifications [35, 36]. Path 2 is promoted via acid-catalysed opening of the fused aziridine ring [25] to give the C-1 carbonium ion (**14**). Extensive charge delocalization by a QM-like intermediate allows **14** sufficient lifetime to react with cellular nucleophiles to ultimately form N2-guanine/N2-guanine interstrand and intrastrand bifunctional DNA adducts and the decarbamoyl N2-guanine monoadduct (path 2, Fig. 1) [37, 38]. Deprotonation of **14** followed by enol/keto tautomerization of the QM (**15**) yields 2,7-DM (**6**, path 3). The presence of high amounts of 2,7-DM in enzyme incubations [29], cancer cells [30], and solid tumours [39, 40] after MMC treatment confirms path 3 as a major contributor in MMC metabolic activation. 2,7-DM can then undergo further quinone reduction to generate a unique covalent modification identified as the N7 guanine major groove DNA adduct [31, 41]. The pH dependence of path 2 explains the observation that at more acidic pH the activity of MMC increases [42, 43], since this is coupled to an increase in formation of the more cytotoxic DNA cross-links [44–46]. Nevertheless, a recent study suggests that monoadducts are equally as toxic as cross-linked adducts in certain cell lines [47].

The key factors that promote bifunctional DNA alkylation and, therefore, increased activity are:

1. fast reducing conditions, which consume parent compound,
2. acidic pH, and
3. very low overall MMC concentrations [48].

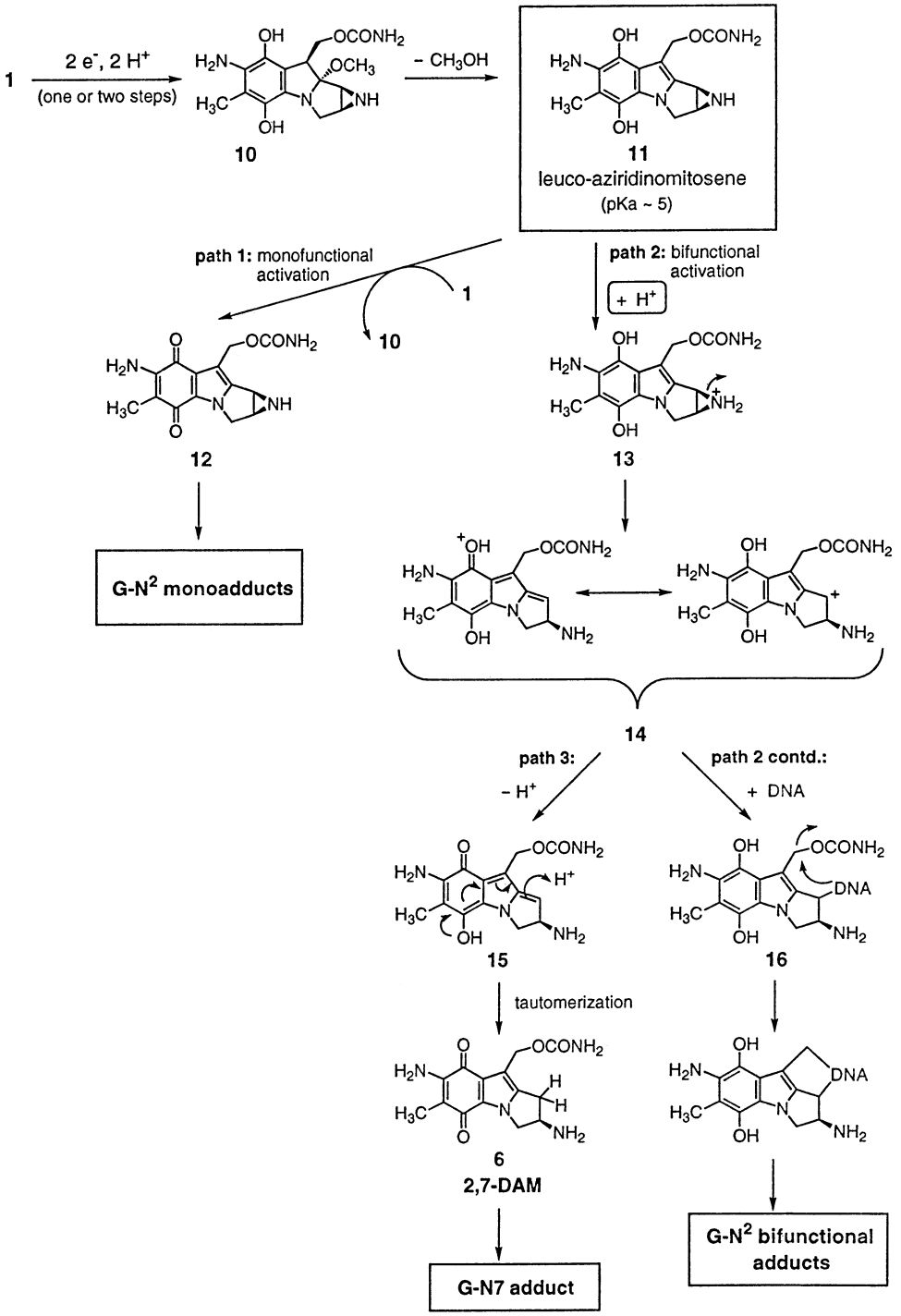

FIG. 1. Pathways of metabolic activation of mitomycin C leading to covalent modification of DNA (adapted from Ref. 26). The three competing pathways are fully explained in the text.

These conditions may be more favoured *in vivo* in solid tumours, where only low levels of drug are likely to penetrate and reductive enzyme activity may be very high [26]. The observation that the N2-guanine/N2-guanine interstrand cross-link adduct was the major DNA adduct produced in EMT6 cells after exposure to MMC [49] supports this opinion.

There have been few studies detailing the identity of DNA covalent adducts formed after *in vivo* drug administration [50]. The pattern of MMC DNA adducts produced in the tissues of non-tumour-bearing rats treated with 9 mg/kg of MMC has been studied using [^{32}P]-postlabelling [51]. Interestingly, an even tissue distribution of 10 adducts was observed. One major product accounted for 71% of all adducts. Using extracted rat liver DNA and a series of homo-oligonucleotides (poly dG, poly dC, poly dA, and poly T) as standards, it was shown that greater than 90% of *in vivo* adducts were with guanine. Although the major *in*

vivo adduct and *in vitro* adduct proved to be different, these data confirm *in vivo* the remarkable specificity of MMC for guanine.

ENZYMOLOGY OF MMC METABOLIC ACTIVATION *IN VITRO*

Thus, metabolic activation of MMC by both chemical reductants ($Na_2S_2O_4$ and H_2) and important bioreductive enzymes (DT-diaphorase, NADH cytochrome *c* reductase, and NADPH cytochrome *c* reductase) occurs via the same general scheme (Fig. 1), regardless of the source of reducing equivalents [26]. Partitioning into the various pathways (and therefore the profile of DNA adducts generated and, hence, anticancer activity) is dictated more by chemical considerations, conditions of pH, and drug concentration than by the nature and properties of the agent performing the initial reduction. There was one important exception to this general rule; the enzyme DT-diaphorase produced fewer DNA adducts than expected at neutral pH compared with other treatments, confirming previous observations of inactivation of this enzyme by MMC at neutral pH [30, 52]. An orientation model has been proposed to explain this phenomenon where the close proximity of MMC (or possibly 7-aminoaziridinomitosene) to nucleophilic residues in the active site of the protein results in autoalkylation [53].

A growing catalogue of proteins has been demonstrated to catalyze bioreduction of MMC *in vitro*. These include cytochrome P450 reductase [29, 33], xanthine oxidase [29, 33], and cytochrome b_5 reductase [54] acting as one-electron reductases, and DT-diaphorase [55] and xanthine dehydrogenase [56] acting as two-electron reductases. Carbonyl-reductase does not appear to metabolize MMC [57], and cytochromes P450 are reported not to transfer electrons directly to MMC but may facilitate quinone reduction at least two-fold through drug binding [58]. However, studies in rat hepatocytes show that cytochromes P450 can reduce MMC to a semiquinone free radical [59]. A unique cytosolic activity capable of metabolic activation of MMC is believed to be present in a number of cell lines [60]. The enzyme exhibited properties of a diaphorase but was distinct from NQO1, cytochrome P450 reductase, and cytochrome b_5 reductase. It was speculated to be an unknown isozyme or a heterodimeric form of NQO1. There has been a paucity of studies on MMC metabolism by mitochondrial enzymes [61], although the high degree of toxicity of the drug to mitochondria may suggest site-specific metabolic activation [62].

The hierarchy of participation from each of these reductases to metabolic activation of MMC in intact cells has still to be established, and this clearly remains a major goal for the successful application of the enzyme-directed approach to anticancer drug development. Under different physiologic conditions, different enzymes may prevail [3]. Key roles for at least two reductases have been proposed.

Cytochrome P450 reductase traditionally is regarded as the enzyme responsible for metabolic activation of MMC in hypoxic cancer cells [63, 64], although it has also been claimed that this reductase contributes to the cytotoxicity of MMC only in normally oxygenated CHO cells, where presumably the main cellular toxins produced are ROS as compared with alkylating moieties [65]. Utilizing CHO cells transfected with human cytochrome P450 reductase, resulting in a 10- to 20-fold increase in activity, the role of this enzyme has been clarified. Only a modest increase in cytotoxicity (2.9- to 4.9-fold) occurred under aerobic conditions [66] but a much larger increase occurred under anaerobic conditions [67], confirming the leading position of this enzyme in hypoxic cells. Nonetheless, such investigations should be viewed with a certain degree of caution since the level of other reductases present, such as DT-diaphorase (which was low) and xanthine oxidase/dehydrogenase (which was absent), can dictate to a large extent the outcome of these experiments (see below).

DT-diaphorase is generally held as the enzyme responsible for bioactivation of MMC in normally oxygenated cells [55, 68–70]. In direct contradiction, data generated with pure enzyme indicate minimal or no metabolic activation and DNA cross-linking at physiological pH by DT-diaphorase [30, 71–73]. While MMC is undoubtedly a poor substrate for DT-diaphorase [74], physiological conditions present in cells (low drug concentrations/high reductase levels/more acidic pH) but absent in cell-free systems may combine to enhance the ability of DT-diaphorase to activate MMC [26]. This could resolve the above contradictory data. It is now clear that DT-diaphorase, either as the pure protein, in cells possessing high levels of the enzyme, or in CHO cells transfected with the enzyme, can transform MMC to cytotoxic species under both aerobic and hypoxic conditions with equal facility [20, 75]. Nonetheless, reports continue to be published—in this case using CHO transfectants—maintaining that MMC is not a substrate for this enzyme [76]. However, publications supporting a major role for this enzyme in MMC metabolic activation in cancer cells are, by far, in the majority. These have been extended from CHO cells to a panel of human colon and gastric carcinoma lines and a series of transfectants derived from a parental gastric carcinoma cell line [77]. Significantly, in that study increased binding of MMC to DNA was demonstrated, showing convincingly that not only does overexpression of DT-diaphorase contribute to increased cytotoxicity, but it also promotes metabolic activation.

An important phenomenon, with major implications for the role of DT-diaphorase in MMC metabolic activation, has been observed in cells treated with the DT-diaphorase inhibitor DIC. Exposure to DIC, curiously, often results in an increase in the cytotoxicity of MMC, especially in hypoxic cells rich in the enzyme [49, 58, 78, 79]. These findings have been explained by a mechanism where different bioreductive enzymes compete for MMC as a substrate on the basis of protein levels [3]. When in excess, DT-diaphorase will be successful over other species but, being less efficient at metabolic activation because of

pH-dependent inactivation, will ultimately result in reduced cytotoxicity [52, 53]. The lack of selectivity is believed to be due to the fact that most major bioreductive enzymes exhibit very low Michaelis–Menten affinity constants for MMC. Xanthine oxidase and cytochrome P450 reductase have a K_m of 2 mM [29]; xanthine dehydrogenase, a K_m of 299 μM [80]; and DT-diaphorase, a K_m of 1 mM [73]. Inhibition of DT-diaphorase with DIC allows other more efficient enzymes to contribute and, hence, the increase in activity. In cell lines high in DT-diaphorase, little differential in cytotoxicity is noted between aerobic and anaerobic conditions, consistent with the predominant role for the enzyme [74, 75, 78, 79], whereas in cell lines low in diaphorase a greater differential is recorded due to a predominant role for one-electron reductases such as cytochrome P450 reductase [63, 78, 79]. Initial observations on hypoxic cell selectivity of MMC were made in EMT6 cells where cytochrome P450 reductase, a one-electron reductase, was believed to be responsible for catalysing the majority of metabolic activation [42, 63]. On a cautionary note, data generated using high concentrations of DIC can be misleading for several reasons [78, 79]. DIC can inhibit cytochrome b_5 reductase [53] and cytochrome P450 reductase [81], stimulate xanthine dehydrogenase [82], induce the formation of DNA adducts [49], and inhibit a unique cytosolic diaphorase-like activity [60].

Thus, DT-diaphorase occupies a central role in the metabolic activation of MMC because it can be present at very high levels in cell lines and human tumour specimens [13–16]. Expression of DT-diaphorase is under the control of several transcription factor binding elements present in the 5′ promoter region of the NQO1 gene such as the antioxidant response element (ARE), the xenobiotic response element (XRE), and the AP-2 element [83–85]. These are activated by transcription factors such as AP-1 and NF-κB, which, in turn, are induced by redox-active drugs, hypoxia, oncogene expression, and drug treatment with MMC itself [84, 86, 87].

CORRELATION BETWEEN ENZYME ACTIVITY AND *IN VITRO* ACTIVITY

In view of the complexity of the enzymology and pathways of MMC metabolic activation (see above and Fig. 1), it might be more appropriate to ask whether it is even realistic to expect there to be a clean correlation between the activity of one bioreductive enzyme and *in vitro* chemosensitivity. The recent elucidation of several biochemical and cellular factors that are of equal importance to metabolic activation and DNA damage in dictating cytotoxicity to MMC only adds to the complexity and difficulty in defining single critical determinants of drug activity [88–91].

Studies utilizing panels of cell lines that offer varying contents of bioreductive enzymes have tended to be performed under aerobic conditions. Not surprisingly, no correlation is observed between the catalytic activity of one-electron reductases such as cytochrome b_5 reductase and cytochrome P450 reductase and cytotoxicity, because generation of alkylating moieties by these enzymes is inhibited by oxygen [77, 92]. Data are more encouraging with DT-diaphorase, where a highly significant correlation ($r^2 = 0.424$, $P < 0.0005$) was reported between enzyme activity and MMC sensitivity in the National Cancer Institute (U.S.A.) panel of 69 human tumour cell lines [92]. By contrast, no correlation was obtained in a panel consisting of predominately lung cancer cell lines [93].

ENZYMOLOGY OF MITOMYCIN C METABOLIC ACTIVATION IN TUMOUR TISSUE

In an attempt to understand the process of MMC metabolic activation *in vivo*, Spanswick *et al.* studied the metabolism of the drug in material obtained from two MAC solid tumours (MAC 16 and MAC 26) [61]. Tumours were fractionated into cytosols, microsomes, and mitochondria in order to identify enzymes catalysing MMC bioreduction. 2,7-DM was used as a marker metabolite for MMC metabolism and bioreductive activation (see Fig. 1) [40].

In the MAC 16 tumour, activities were localized to all three subcellular fractions, including the first demonstration of MMC metabolism by mitochondria from solid tumours [61]. Characterization of the fractions in terms of co-factor requirements and effects of inhibitors and molecular oxygen revealed the cytosolic activity to be due to DT-diaphorase, while the microsomal activity was attributable to cytochrome P450 reductase. The novel mitochondrial enzyme(s) was shown not to be DT-diaphorase or cytochrome P450 reductase, both of which can be present in mitochondria from tumour cells [67, 94]. The novel enzymes accepted both NADPH and NADH as cofactors (suggesting a diaphorase) but were 100% inhibited by molecular oxygen (indicative of one-electron reduction). It was concluded that the NADPH-dependent enzyme (the major activity) was most likely to be mitochondrial NADPH cytochrome *c* reductase (otherwise known as cytochrome P450 reductase) [95]. While sharing similarities to its microsomal counterpart, it also exhibits differences in terms of molecular weight and peptide elution profile [96], and this may account for the lack of recognition by an antiserum raised against the microsomal enzyme [61]. The NADH-dependent activity could only be speculatively ascribed to either NADH dehydrogenase, which has been linked to the metabolism of the anthracycline doxorubicin [97], or NADH-ubiquinone oxidoreductase [61]. Confirmation of the identity of these novel reductases awaits their purification. In the MAC 26 tumour, MMC bioreduction was catalysed exclusively by the NADPH-dependent mitochondrial enzyme.

Thus, solid tumour tissue can express bioreductive capabilities thought hitherto not to be present in cell lines [77, 92]. Other bioreductive enzyme activities are likely to be present in solid tumours, including known enzymes such as nuclear cytochrome P450 reductase and DT-diaphorase [93, 98, 99] and novel activities yet to be identified [60].

Therefore, metabolic activation of MMC in solid tumour tissue, as in cell lines, can be catalysed by several bioreductive enzymes located at different subcellular locations distributed throughout the cell, each competing for MMC as a substrate.

In addition to identifying individual species, it is very important to the enzyme-directed approach to understand how the different enzymes are likely to behave in a reconstituted tumour. The MAC 16 tumour contains at least three competing sources of enzyme activity (see above). The major activity (3.7 nmol/30 min/mg of protein) was a mitochondrial NADPH-dependent one-electron reductase (believed to be mitochondrial cytochrome P450 reductase) followed by microsomal cytochrome P450 reductase (2.04 nmol/30 min/mg of protein) followed by cytosolic DT-diaphorase (0.76 nmol/30 min/mg of protein) [61]. Whole tumour homogenates, however, behaved more like a preparation of DT-diaphorase: activity was lower than expected and was insensitive to molecular oxygen. In contrast, in the MAC 26 tumour where only the mitochondrial enzyme was detected, tumour homogenates exhibited a much higher level of activity, which was oxygen sensitive, despite the fact that MAC 26 has been shown to be resistant to bioreductive drugs [100].

These data reveal two very important findings. First, DT-diaphorase does appear to be less effective at activating MMC even in a solid tumour homogenate [30, 31, 78, 79]. Second, DT-diaphorase remains capable of competing successfully for MMC as a substrate within the medium of a tumour homogenate even when other more active enzymes are present [3]. As explained above, because none of the major bioreductive enzymes exhibits particularly high affinity for MMC as a substrate, the enzyme present in the largest excess predominates. In the case of MAC 16, this is DT-diaphorase, which is known to be overexpressed in this tumour [61, 100]. Similar results have been obtained recently in two human colon cancer xenografts: HT29 (very high in DT-diaphorase) and BE (DT-diaphorase activity absent) [Cummings *et al.*, unpublished observation].

IMPLICATIONS FOR ENZYME-DIRECTED BIOREDUCTIVE DRUG DEVELOPMENT

The enzyme-directed approach relies heavily on a strong correlation existing between antitumour activity and the level of expression of a single bioreductive enzyme [12]. With an emerging picture of increasing complexity, there are genuine concerns over the feasibility of targeting a drug to a single bioreductive enzyme in human solid tumours [21, 101], especially taking into account additional contributing factors such as cellular heterogeneity, DNA repair processes, antioxidant defenses, physiological factors, and variable oncogene expression [4]. Nonetheless, it is the case that, when there is *marked* overexpression of a particular bioreductive enzyme such as DT-diaphorase, a reasonable correlation may hold *in vitro* [20, 92]. Only limited *in vivo* studies have been carried out with MMC and related bioreductive drugs in solid tumours characterized for bioreductive enzyme contents. An apparent correlation was observed between DT-diaphorase expression and response to MMC in a panel of eight NSCLC xenografts [13]. With the exception of two specimens, all the xenografts expressed high levels of the enzyme >100 nmol/min/mg of protein), and while there was a 10-fold variation in enzyme activity within this group, chemoresponsiveness did not vary by the same degree. It is interesting to note that MMC is recognized as one of the most active single agents in the treatment of NSCLC in humans [102]. In direct contradiction, two *in vivo* studies have reported a negative correlation between DT-diaphorase activity and the antitumour activity of MMC [103, 104]. Furthermore, in a panel of human g.i. tract xenografts that expressed very high levels of the enzyme (299–1707 nmol/min/mg protein), co-administration of DIC resulted in a greater increase in activity in the tumours expressing the highest level of enzyme [104]. These data are more analogous to the *in vitro* situation in hypoxic cells and are consistent with DIC eliminating less efficient metabolic activation by DT-diaphorase in favour of more efficient metabolic activation by one-electron reductases.

Numerous studies with the closely related MMC analogue indoloquinone EO9 have demonstrated a complete lack of correspondence between DT-diaphorase activity and *in vivo* antitumour activity [21, 105]. Notwithstanding this fact, EO9 is a much better substrate for the enzyme than MMC, and its activity *in vitro* correlates very closely to DT-diaphorase expression [73, 74, 105–107]. In one of the above *in vivo* studies, there was also no correlation between the antitumour efficacy of EO9 and the activity of other bioreductive enzymes including cytochrome P450 reductase and cytochrome b_5 reductase [21].

Another example of a related bioreductive compound is 2,5-dimethyl-3,6-diaziridnyl-1,4-benzoquinone (MeDZQ), which is one of the best known substrates available for recombinant human DT-diaphorase [74]. Its *in vitro* cytotoxicity increased up to 4-fold in CHO cells transfected with human NQO1 cDNA, confirming metabolism by the enzyme in whole cells [76]. However, in a panel of human NSCLC xenografts, antitumour activity did not correspond to DT-diaphorase or cytochrome P450 reductase activity [108]. A much better correlation has been observed for EO9 and MeDZQ when the overall capacity of the tumour to metabolize the drug was taken into account [21, 108]. No assumptions are made regarding the identity or exclusivity of the enzyme(s) responsible for metabolic activation by adopting this approach, which probably represents a composite measurement including contributions from many different processes [21]. The assay involved a simple *in vitro* incubation of the bioreductive drug with a homogenate of a tumour specimen. Drug metabolism was followed by HPLC analysis as the rate of disappearance of the parent drug [21].

In summary, the enzyme-directed approach to drug de-

sign, as originally formulated [12], may require remodelling in the light of recent findings, especially from the results of a series of *in vivo* experiments where only poor correlations were observed between enzyme expression and antitumour activity. An alternative approach is available, which involves a determination of the ability of a biopsy to metabolize the drug, providing it can be established that the assay measures metabolic activation [21]. A new universal mechanism of action for MMC is proposed consisting of three competing pathways, which result in different DNA adduct profiles and end products, and which are governed principally by chemical and physiological considerations. Metabolic activation of MMC by solid tumours can be catalysed by numerous different enzymes. However, metabolism will be carried out predominately by the bioreductive enzyme present in greatest excess, because most reductases studied to date show very little specificity for the drug.

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