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Review

Tailoring Targeted Therapy to Individual Patients: Lessons to be Learnt from the Development of Mitomycin C

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Abstract. *The modern era of targeted therapeutics offers the potential to tailor therapy to individual patients whose tumours express a specific target. Previous attempts to forecast tumour response to conventional chemotherapeutics based on similar principles have however been disappointing. Mitomycin C (MMC), for example, is a bioreductive drug that requires metabolic activation by cellular reductases for activity. The enzyme NAD(P)H:Quinone oxidoreductase-1 (NQO1) can reduce MMC to DNA damaging species but attempts to establish the relationship between tumour response to MMC and NQO1 expression have generated conflicting reports of good and poor correlations. Several other reductases are known to activate MMC. This, in conjunction with the fact that various physiological and biochemical factors influence therapeutic response, suggests that the mechanism of action of MMC is too complex to allow tumour response to be predicted on the basis of a single enzyme. Alternative approaches using more complex biological and pharmacological systems that reflect the spectrum of reductases present within the tumour have been developed and it remains to be seen whether or not the predictive value of these approaches is enhanced. With regards to targeted therapeutics, the experience with MMC suggests that prediction of tumour response based on analysis of a single target may be too simplistic. Multiple mechanisms of action and the influence of tumour*

microenvironment on cell biology and drug delivery are likely to influence the final outcome of therapy. The challenge for the future progression of this field is to develop assays that reflect the overall biological and pharmacological processes involved in drug activation whilst retaining the simplicity and robustness required for routine chemosensitivity testing in a clinical setting.

The ability to tailor chemotherapy to individual patients who are most likely to benefit from treatment has been a major objective in cancer research for many years. Since the seminal studies of Hamburger and Salmon in the late 1970's where primary cell cultures and clonogenic assays were used (1), numerous approaches have been evaluated. Despite extensive research, the ability to accurately forecast the response of individual patients to chemotherapy remains elusive. The modern era of targeted therapeutics offers enhanced prospects of individualised therapy. The development of trastuzumab for use against HER-2 positive breast cancers is a good example of this (2, 3). Whilst the example of trastuzumab represents a significant step forward individualised therapy, is it realistic to expect that all tumours that express a specific target will respond to target orientated therapies? Even in the case of trastuzumab, tumour response remains heterogeneous with some patients responding well whereas others are inherently resistant to treatment (4). Numerous attempts have been made to predict the response of tumours to conventional cytotoxics using similar approaches. However, no reliable assay is currently in routine clinical use. In this article, the predictive value of assays designed to forecast the response of tumours to mitomycin C (MMC, Figure 1) will be critically reviewed. The rationale for selecting MMC is that it is a targeted anticancer agent and extensive efforts have been made to predict tumour response based upon analysis of key enzymes involved in its mechanism of action. The principle objective of this review is to obtain an understanding of why previous approaches have been unsuccessful in the belief that this may inform the design of predictive assays for targeted therapeutics.

Abbreviations: MMC: Mitomycin C; NQO1: NAD(P)H:Quinone oxidoreductase-1; ROS: reactive oxygen species; SNP: single nucleotide polymorphism; 2,7-DAM: 2,7-diaminomitosen; BSO: L-buthionine-R,S-sulfoximine.

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MMC and the Potential for Tailored Therapy

MMC is a naturally occurring antibiotic that was isolated from *Streptomyces caespitosus* in 1956 by Hata *et al.* (5). It belongs to a class of anticancer agents known as bioreductive drugs and it is regarded as the prototypical bioreductive drug (6). MMC is a clinical component of combination chemotherapy regimens used to treat a range of tumour types (7). Throughout Europe, MMC is routinely used as a single agent to treat superficial bladder cancers and it significantly delays tumour recurrence (8, 9). The response of superficial bladder cancers to MMC is heterogeneous (10). So there is a clinical need to identify patients that are most likely to benefit from treatment.

In general terms, bioreductive drugs are molecules that require metabolic activation by cellular reductases to generate metabolites that damage DNA. They were initially developed to target hypoxic cells and selectivity was determined by the ability of oxygen to reverse the activation process (11, 12). As our understanding of the enzymatic activation processes increased, it became clear that certain bioreductive drugs can also target aerobic cells (13). In this case, selectivity is determined primarily by the presence of elevated levels of reductase activity in tumour tissue (13). This generated the concept of 'enzyme directed bioreductive drug development', an integral component of which is the ability to forecast tumour response based upon knowledge of the activity of specific reductases and/or the extent of tumour hypoxia (14). Whilst conceptually elegant, its ultimate value depends upon a number of conditions. The main requirement is that one enzyme plays a dominant role in bioreductive activation and that tumour response is determined by the expression of this enzyme in tumours. Furthermore, it also assumes that cells ability to respond to DNA damage (DNA repair or ability to undergo apoptosis) play a comparatively minor role in determining response. The ability to predict tumour response based upon analysis of a single enzyme and approaches that challenge the assumptions outlined above will, therefore, be the initial focus of this review.

MMC and its Mechanism of Action

The mechanism of action of MMC has been extensively studied and reviewed elsewhere (15-18). Briefly, the initial and critical step in the bioactivation process is reduction of the quinone to either the semiquinone or the hydroquinone by one- or two-electron reductases, respectively (Figure 1). In the presence of oxygen, the semiquinone enters into a redox cycle that produces reactive oxygen species (ROS) which can lead to DNA damage. It is generally acknowledged, however, that the DNA damage caused by ROS contributes little to the overall cytotoxicity of MMC

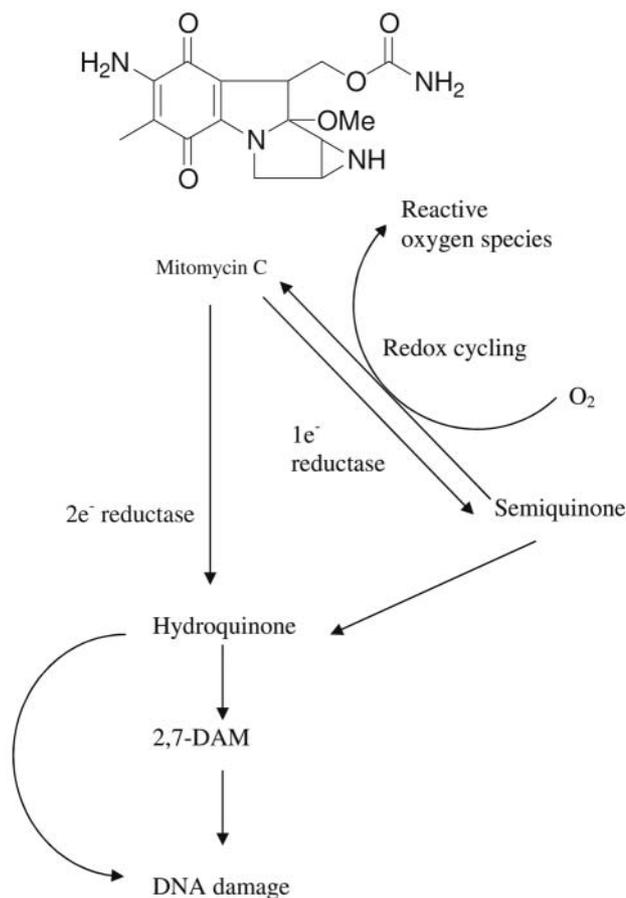


Figure 1. Basic schematic of MMC bioactivation. For a more complete overview of the full metabolic route, see Cummings *et al.* (17).

compared to DNA alkylation (19, 20). Under hypoxic conditions, the semiquinone free radical chemically rearranges into the more stable hydroquinone which undergoes a series of chemical reactions leading ultimately to alkylation of DNA (21). When the hydroquinone species are generated directly by 2 electron reductases (by passing the semi-quinone), the generation of alkylating species is not dependent upon the presence or absence of oxygen. The covalent DNA adducts produced are predominantly formed at the N2 position of guanine (but also at the N7 position) and both intra-strand and inter-strand crosslinks as well as mono-adducts are produced. It is however the interstrand cross links rather than the monoadducts that are critical factors in determining cell death (22).

Physiological conditions, such as low pH (23, 24) and oxygen tension (25, 26) can have a significant influence on cellular response to MMC but the key step in the process remains the initial reduction of the quinone nucleus by one and/or two electron reductases. Numerous enzymes have been implicated in the reduction of MMC. These include cytochrome P450 reductase (27), xanthine dehydrogenase

(28), xanthine oxidase (29, 30), NADPH ferredoxin reductase (31), cytochrome b5 reductase (32), an uncharacterised cytosolic enzyme (15, 33), glucose regulatory protein 58 (34, 35), HAP1 (35), NQO1 (36) and more recently NAD(P)H:Quinone oxidoreductase 2 (37, 38). Whilst MMC is a substrate for many enzymes, attention has predominantly focused on NQO1 (17, 18, 39) and the relationship between NQO1 and response to MMC has been extensively studied. In addition to enzymatic reduction, a series of other events should be taken into consideration as these may also determine cellular response. These are summarised in simplified form in Figure 2 and include; reduction of MMC by reductases and other metabolic routes such as detoxification pathways, the formation of DNA damage and its repair, and finally the induction of cell death. Similar reasoning could be extended to other forms of targeted therapy in terms of the presence of an active target, the target/drug interaction, the effect of this interaction (*e.g.* activation or repression of a signalling pathway, tubulin disruption) and the induction of cell death. Each step of this process may be of importance in predicting response to treatment and will be commented upon in this review. Initially however, the relationship between NQO1 and response to MMC will be discussed.

Role of NQO1 in MMC Bioactivation and the Prospect of Individualising Therapy Based on NQO1 Expression

NQO1 is a cytosolic flavoprotein that catalyses the two-electron reduction of a broad range of substrates (particularly quinone based compounds) using NADH or NADPH as the source of electrons (18, 40, 41). Several possible physiological functions for NQO1 have been proposed (42, 43) although historically, its role in protecting cells from highly reactive environmental and synthetic quinones is believed to be its principle function. This is achieved by converting quinones directly into redox stable hydroquinones, thereby bypassing the semi-quinone free radical and subsequent generation of ROS caused by redox cycling in air. The newly formed hydroquinones can be conjugated to glucuronides or sulfides, resulting in increased water solubility and excretion (44). In the case of certain quinones however, the hydroquinone may itself autoxidise to generate ROS or directly alkylate DNA. In these cases, reduction by NQO1 represents a pro-drug activation mechanism. The decision as to whether a compound is detoxified or activated by NQO1 is determined largely by the chemistry of the groups attached to the quinone nucleus (44). In the case of MMC, it is a substrate for NQO1 and reduction by NQO1 results in DNA alkylation in cell free assays (36, 45, 46). This fact alone suggests that NQO1 could play a significant role in determining therapeutic response.

Two additional pieces of evidence significantly enhanced the prospect of predicting response to MMC based on analysis of NQO1. First, a series of studies demonstrated that the expression of NQO1 (at the mRNA and protein level) was not only elevated in several tumour types but that within each pathological group of tumours, wide interpatient heterogeneity existed (47-49). Some patient's tumours expressed high levels of NQO1 activity whereas others were devoid of activity, making it conceivable that heterogeneity in tumour response to MMC could be related to heterogenous NQO1 expression. Second, the gene encoding for NQO1 is polymorphic and two single nucleotide polymorphic (SNP) variants (C609T [NQO1*2] and C465T [NQO1*3]) are associated with total loss or significant reductions in NQO1 activity (50-53). In view of the fact that NQO1 can activate MMC, wide variations in NQO1 activity or the presence of SNP's leading to reduced NQO1 activity could have a significant bearing on therapeutic outcome (41). It should be stressed however that the ability of NQO1 to reduce MMC is not simple as it only occurs under mild acidic conditions (45). At physiological pH values, MMC is not only a poor substrate for NQO1 but actually inhibits NQO1 activity (23, 45, 54). Whilst tumours are known to contain regions of low extracellular pH 5.5, intracellular pH is generally maintained at or slightly above neutrality (56). These findings may impact on the relevance of NQO1 as a major determinant of MMC activity as discussed below.

Relationship between NQO1 and Response to MMC *In Vitro*

As stated previously, the existence of a correlation between reductase expression (mRNA and/or protein) and treatment response is an essential requirement for enzyme directed drug development. Several experimental approaches have been employed but as described below, conflicting reports of good and poor correlations between NQO1 expression and response *in vitro* can be found within the available literature. In studies using panels of cell lines that inherently express a broad range of NQO1 mRNA and/or protein, reports of good correlations between NQO1 activity and response (57-61) contrast sharply with reports of poor correlations (15, 62-64). Similarly, enhanced activity of MMC has been observed by some groups in isogenic cell lines where NQO1 has been over expressed (65-68) whereas other laboratories have demonstrated that MMC sensitivity is independent of NQO1 activity in similar models (15, 69, 70). Several studies using cell lines that are resistant to MMC have demonstrated that NQO1 activity is reduced compared to the parental lines (58, 71). Other studies have demonstrated that cisplatin resistant A2780 cells (which have elevated levels of NQO1) are also resistant to MMC,

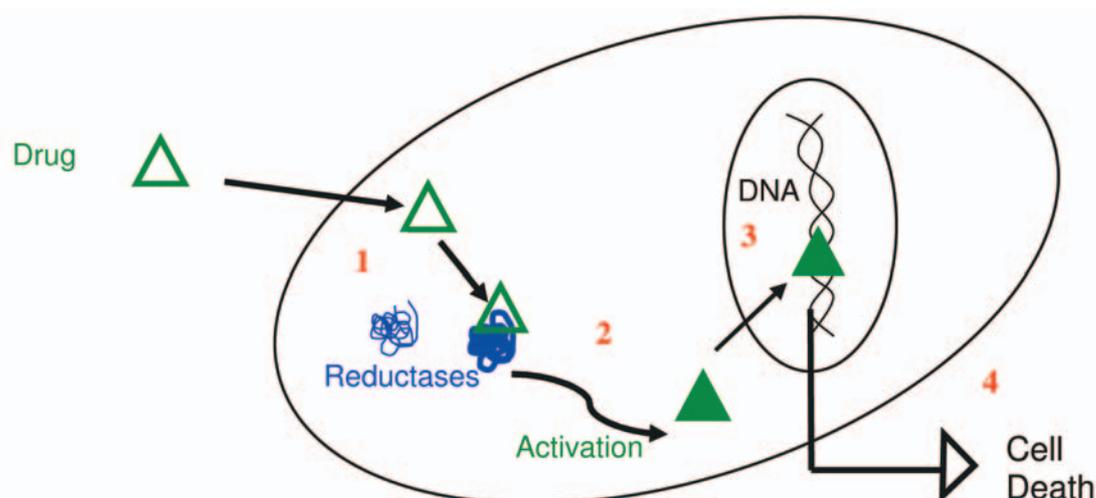


Figure 2. Simplified cartoon outlining the key steps required for cellular response to MMC. 1; presence of reductases/target protein, 2; MMC metabolism or drug/target interaction, 3; DNA damage or effect of target disruption, 4; Cell death induction.

which suggests that other factors influence the outcome of MMC treatment (72). Attempts to chemically modulate NQO1 activity in cell lines, using the inhibitor dicumarol for example, have suggested that NQO1 plays a role in activating MMC (73, 74). Similarly, NQO1 activity can be induced in cells and enhanced toxicity to MMC has been observed following NQO1 induction by 1,2-dithiole-3-thione, dimethyl fumarate and arsenite (75-77). However, caution needs to be exercised in the interpretation of these experiments as dicumarol is not a selective inhibitor of NQO1 (78) and the recent finding that NQO1 can stabilise p53 (79, 80) suggest that manipulation of NQO1 levels in cells could potentially modulate therapeutic response to DNA damage.

The majority of the studies mentioned above have focused on the relationship between NQO1 and response to MMC under aerobic conditions but the situation becomes more complex when similar experiments are conducted under hypoxic conditions. The determination of hypoxic cytotoxicity ratios (HCR; defined as the ratio of IC_{50} values in air divided by IC_{50} values under hypoxia) is a commonly used method to compare the activity of drugs under both aerobic and hypoxic conditions. In the case of MMC, high HCR values have been reported in cell lines that have low or no NQO1 activity (26, 81). In NQO1 rich cells, HCR values are typically very low, indicating that these cells are equally sensitive to MMC in the presence and absence of oxygen (26, 82). Whilst the mechanistic basis for these observations is not fully understood, these results present a significant additional challenge to predictive drug testing for MMC sensitivity based on the analysis of NQO1 alone.

With regards to the potential impact of NQO1 SNP's on MMC sensitivity, resistance to MMC has been demonstrated in a number of cell lines that are homozygous for the NQO1*2 allele (53, 83). Similarly for the NQO1*3 allele, cells are more resistant to MMC than wild-type cells (84, 85). Furthermore, the response of primary cell lines derived from gastric tumours to MMC was dependent upon NQO1 genotype, with increased sensitivity to MMC observed in cells with wild-type NQO1 (86). In contrast to studies based on NQO1 phenotypic expression or activity, no studies have been published that contradict these findings and experimentally at least, genotype status provides the strongest evidence that NQO1 plays a role in determining therapeutic outcome to MMC treatment.

Relationship between NQO1 and Response to MMC in Xenografts and Patients

The ability to predict response to MMC based upon analysis of NQO1 phenotype and genotype has been critically addressed in a series of *in vivo* and clinical studies. With regards to NQO1 phenotype, a good correlation between MMC sensitivity *in vivo* and NQO1 activity was reported in a panel of eight non-small cell and small cell lung tumour xenografts (48). In other studies, however, a poor correlation between anti-tumour activity and NQO1 levels has been reported (87-89). In an extensive panel of 45 human tumour xenografts expressing a broad spectrum of NQO1 enzyme activity, no correlation was observed between NQO1 activity and response to MMC *in vivo* (64). In clinical studies, promising results were obtained by Gan *et al.* where the response of histocultures derived from 21 human bladder

cancer patients (with both superficial and invasive disease) correlated with the mRNA expression of NQO1 and cytochrome P450 reductase (90). In contrast, NQO1 protein expression as measured by immunohistochemistry in 92 superficial bladder cancers (pathology was restricted to G1/G2 and pTa/T1 tumours) did not correlate with clinical response to MMC in terms of time to first recurrence (91).

With regards to the polymorphic variants of NQO1, a study by Fleming *et al.* reported that in 117 patients with disseminated peritoneal cancer treated with intraperitoneal hyperthermic MMC, patients that were heterozygous or homozygous with respect to NQO1*2 had reduced survival compared to patients genotyped as wild-type (92). This very significant finding suggested for the first time that screening for this polymorphism may be useful in identifying individuals who may be at risk of treatment failure following intraperitoneal MMC therapy. In contrast to these encouraging reports, studies in both human tumour xenografts (93) and superficial bladder cancer patients (94) have demonstrated that genotyping individuals with respect to the NQO1*2 SNP did not correlate with therapeutic outcome.

In summary, the ability to forecast the response of tumours based on the levels of NQO1 or NQO1 genotype status is unlikely to be of practical value in the clinic. Whilst some evidence of positive correlations have been reported, there is a wealth of conflicting data in the literature which suggests otherwise. A number of other enzymes have been implicated in the activation of MMC and some attempts have been made to correlate their expression status, sometimes in conjunction with the NQO1 levels, with response to MMC treatment. It remains to be seen whether the response of tumours to MMC can be forecast based on the expression of these enzymes but, the complexity of MMC activation suggests that this approach is unlikely to be productive. Models or assays that mimic more closely the complexity of the biological system offer a potential way forward and some of these approaches are discussed in the following sections.

Analysis of MMC Metabolism in Crude Tumour Homogenates; Relationship with Cellular and Tumour Response to MMC

As multiple enzymes are involved in the activation of MMC, Cummings *et al.* proposed an alternative approach based upon the ability of tumour homogenates to metabolically activate MMC (17). This represented the first move from single enzyme/marker prediction to multiple enzyme activity assessment and it is based on the rationale that tumour homogenates will contain a 'cocktail' of reductases capable of activating MMC. By using analytical techniques identifying active metabolites, a more accurate assessment of a tumour's ability to activate MMC may therefore be

achieved (17). Initial studies focused on the identification of 2,7-diaminomitosene (2,7-DAM) as an indicator of bioreductive activation. 2,7-DAM is a transient intermediate formed after the reduction of MMC and it is used as a marker of drug activation (Figure 1). In two murine colon carcinoma models (MAC 16 and MAC 26) 2,7-DAM was readily detected in tumours following intra-tumoral injection of MMC (15). However, higher levels of 2,7-DAM were detected in the comparatively more resistant MAC 26 tumour model compared to MAC 16 tumours, indicating that other pharmacological factors have a significant influence of therapeutic outcome. The analysis of 2,7-DAM is however technically challenging as it is a reactive metabolite that readily binds covalently to cellular macromolecules and once bound, it will be invisible to the majority of analytical techniques.

In an attempt to circumvent this issue, studies conducted in our laboratory analysed the ability of tumour homogenates from a panel of resistant and sensitive human tumour xenografts to metabolise MMC (64). The rate of metabolism of the parent MMC compound was significantly greater in the MMC sensitive group ($t_{1/2}=75\pm48.3$ min, $n=6$) compared to the resistant group ($t_{1/2}=280\pm129.6$ min, $n=11$). Based upon these results, a pilot study was established in 30 patients with superficial (G1/G2 and pTa/T1) bladder cancer where surgical specimens were immediately flash frozen in liquid nitrogen and transferred to the laboratory. Using identical methodologies to our previous studies (64), this study demonstrated that the ability of tumour homogenates to metabolise MMC varied considerably between individual tumours with half lives ranging from 17.5 min to >1000 min (Figure 3).

The key question was whether or not these inherent differences in the ability of tumours to metabolise MMC translates into differences in therapeutic response. Following tumour resection, each patient received a single dose of MMC (40 mg/40 ml) administered intravesically within 24 hours of surgery and time to first recurrence was determined by established follow up procedures. No trend could be established between either metabolism rates and time to first recurrence or metabolism rates and the presence or absence of recurrent disease at the time of survey. Surprisingly, some tumours that slowly metabolised MMC *in vitro* responded as well to MMC as tumours that rapidly metabolised MMC (patients A and B, Table I). Within the G2/pTa tumours, 3 patients suffered recurrence following MMC therapy. Patients C and D, had similar clinical response (first recurrence 3 months after MMC therapy) despite the fact that tumour homogenate from patient C metabolised MMC rapidly ($t_{1/2}=17.5$ min) whereas tumour homogenate from patient D metabolised MMC more slowly ($t_{1/2}=395$ min) (Table I). The observation that several of the tumours that

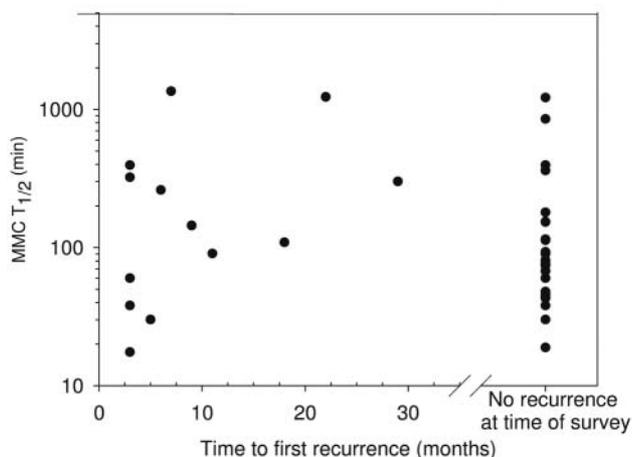


Figure 3. Relationship between MMC metabolism by bladder tumour homogenates and clinical response to MMC. Protein homogenates (10 mg/ml) were then incubated at 37°C in the presence of 2 mM NADH and 2 mM NADPH and the reaction was initiated by the addition of MMC (200 µM). At various time intervals, 30 µl of the reaction mixture was removed and added to 90 µl of acetonitrile containing the internal standard, porfiromycin (PFC, 50 µM). Samples were evaporated and resuspended in mobile phase. Chromatographic separation of MMC by HPLC has been described previously (64). Detection of MMC is performed at 365 nm and 310 nm with a flow rate of 1 ml/min. The half-life of MMC was determined from least squares log linear regression analysis $t_{1/2}=0.693/Kd$ where Kd is the decay rate constant (the slope of the regression analysis $\times 2.303$).

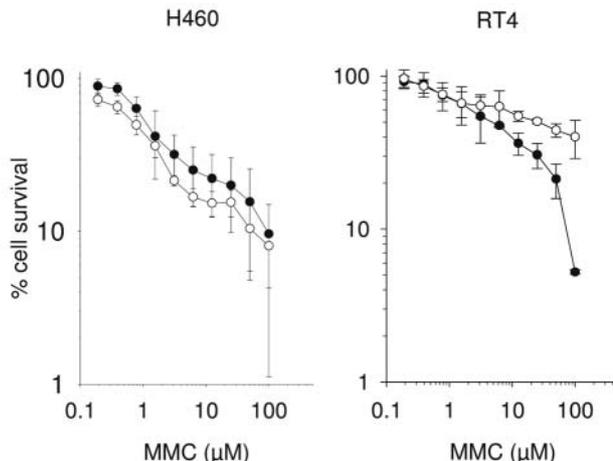


Figure 4. The influence of the glutathione detoxification pathway on MMC cytotoxicity in human cancer cell lines. The effect of the glutathione detoxification pathway was evaluated, using BSO, an inhibitor of glutathione synthesis, reported to be effective when used at 50 µM for 24 h (118). Cells were plated into 96 well culture plates incubated overnight. The following day, cells were incubated with BSO (50 µM) or fresh medium for 24 h. Cells were then exposed to a range of MMC concentrations (0.2 µM to 100 µM) for 1 h in absence of BSO, after which they were washed twice with HBSS prior to addition of complete media. Cell survival was assessed using the MTT assay. IC_{50} values are expressed in terms of the mean \pm standard deviation for three independent experiments. MMC cytotoxicity in H460 and RT4 cells pre-treated with 50 µM BSO (●) for 24 h or in absence of BSO (○). Each data point represents the mean \pm SD for 3 independent experiments.

Table I. MMC half-life and clinical response obtained for 4 superficial bladder cancer patients treated with MMC. These patients were selected to illustrate the extremes of both clinical response to MMC and MMC metabolic rates.

| Patients | Tumour grade | MMC half-life (min) | Time to first recurrence (month) |
|----------|--------------|---------------------|----------------------------------|
| A | G1/pTa | 93 | >27 |
| B | G1/pTa | 853 | >24 |
| C | G2/pTa | 17 | 3 |
| D | G2/pTa | 395 | 3 |

metabolised MMC slowly responded well to treatment (Table I) implies that only a small fraction of MMC is needed to be activated to DNA damaging species or that the tumour cells themselves are exquisitely sensitive to low levels of DNA damage. On the other hand, several tumours were able to metabolise MMC rapidly but failed to respond well to treatment (Table I). This suggests that metabolism of MMC is not only a reflection of bioreductive activation but also of other metabolic routes such as detoxification pathways.

The glutathione-glutathione S transferase pathway is a known resistance mechanism for MMC (95). To exemplify this, additional studies in our laboratory have analysed the effect of glutathione depletion by the non-toxic agent L-buthionine-R,S-sulfoximine (BSO) on *in vitro* chemosensitivity to MMC. The IC_{50} obtained after treatment of H460 cells with BSO for 24 hours (50 µM) followed by exposure to a range of MMC concentrations for 1 hour, were 1.45 ± 0.91 µM, compared to 0.77 ± 0.28 µM in the absence of BSO (Figure 4). In contrast, exposure of RT4 cells to BSO prior to MMC treatment induced a 6.5-fold decrease in the IC_{50} , from 30.20 ± 7.33 µM down to 4.62 ± 3.80 µM (Figure 4). These results suggested that inherent differences in detoxification pathways (e.g. glutathione) existed between RT4 and H460 cells that could have had a significant bearing on MMC sensitivity.

In summary, whilst significant differences exist in the ability of tumours to metabolise MMC, the lack of correlation between metabolic rates and tumour response suggests that this approach needs to be refined. The ability to measure active metabolites, the role of detoxification pathways and the destruction of tumour microenvironments during the preparation of homogenates represent significant obstacles that would have to be addressed.

Induction and Response to DNA Damage as an Indicator of MMC Sensitivity

Developing the approach outlined by Cummings *et al.* (17), analysis of DNA damage induction in tumour cells would represent not only the end product of bioreductive activation by multiple enzymes but would also take into account the role played by detoxification pathways and other protein/drug interaction. Analysis of DNA damage induction may therefore provide an indication of bioreductive activation. As mentioned previously and discussed in detail elsewhere (16, 17, 22), MMC induces a range of DNA lesions but inter-strand cross links are regarded as the major cytotoxic lesions (22). In a panel of human tumour cell lines, *in vitro* chemosensitivity to MMC correlated well with the induction of DNA inter-strand crosslinks as measured by the comet assay (96). This study demonstrated that analysis of DNA interstrand crosslink formation provides a good indicator of MMC chemosensitivity *in vitro* and further studies are required to assess the relationship between DNA interstrand cross link formation and response to MMC *in vivo* (96).

Two other factors need to be taken into consideration in view of these findings; the ability of cells to repair DNA damage and the response of cells to DNA damage in terms of their ability to undergo apoptosis. With regards to DNA repair, full details of the mechanism of DNA repair of MMC induced DNA damage remains unclear but it involves multiple repair pathways including nucleotide excision repair, homologous recombination repair and translesion bypass repair pathways (97, 98). In contrast to the relationship between interstrand crosslink formation and response, poor correlations between response and DNA repair kinetics (determined by the comet assay) and apoptosis induction (determined by Annexin V-FITC/PI dual staining and Hoescht 33342 staining) were observed (96). Whilst DNA repair processes can generally influence therapeutic response, the data presented in this study suggests that it plays a minor role in determining response to MMC. These findings were in agreement with similar studies analysing the role played by DNA repair in determining response to radiotherapy were no correlation could be established between the two parameters *in vitro* (99-101) and in patients with acute myeloid leukaemia (102). In terms of apoptosis induction, conflicting reports of the relevance of apoptosis to MMC sensitivity have been published. Some studies showed a trend between response to MMC and the levels of apoptosis measured in cell lines and in bladder tumours exposed the drug *ex vivo* (103, 104), whereas other reports showed a lack of correlation between the two parameters (105-108). The latter were in accordance with a review by Okada and Mak describing how DNA damage could trigger processes of senescence or mitotic

catastrophe rather than programmed cell death (109). However, a recent study which used 20 non small cell lung carcinoma samples suggested the existence of a relationship between the levels of apoptosis induced by *ex vivo* exposure to MMC, cisplatin and taxotere and reduced metabolic activity in the cultured samples (110). They highlighted the usefulness of measuring pharmacodynamic parameters of tumour mass cultured and treated *ex vivo* in order to predict response (110).

The correlation observed between inter-strand crosslink formation and response suggested that the process of bioreductive activation remains the major determinant of cellular response to MMC at least *in vitro* and further studies are required to determine whether or not the comet assay can accurately forecast the response of tumours to MMC *in vivo*. The comet assay encompasses the key principles developed above in that it is a cell based assay where bioreductive activation processes take place alongside detoxification pathways in a competitive manner. Recent studies have also demonstrated that the comet assay provides a good indication of response to radiotherapy, is simple, inexpensive, rapid and requires small biopsy samples (99-101, 111). It remains to be seen whether or not the comet assay can be used to accurately forecast the response of superficial bladder cancers to MMC.

Conclusion

Two main issues arise from this review. The first relates to the prospects for developing predictive assays for MMC and the second is what lessons can be drawn from MMC that can or should be considered when developing predictive assays for targeted therapies.

With regards to MMC specifically, there is sufficient uncertainty and conflict in the literature to indicate that analysis of NQO1 alone is unlikely to be beneficial in terms of predicting response. Whether correlations can be obtained between the expression of other reductases involved in MMC activation and response remains to be seen but the complexity of MMC's mechanism of action would suggest that this approach is unlikely to be productive. Cell based assays are a promising way forward as they retain a degree of complexity within the system but this may be compromised by the fact that disruption of solid tumours would also destroy local tumour microenvironments that can influence the activity of MMC. Exposure of biopsies to MMC prior to disaggregating the tissue may circumvent this although this introduces additional problems concerning drug delivery and changes in microenvironmental conditions during drug exposure. Significant obstacles therefore exist in relation to solid tumours and systemic treatments but in the case of superficial bladder cancer, the prospect of developing predictive assays is more promising. As mentioned

previously, MMC is used as a single agent to treat superficial bladder cancer and this simplifies the assessment of clinical response compared to other tumours where MMC is used in combination with other cytotoxics. It is typically administered intravesically within 24 hours of surgery and this provides two key benefits. First, this route of drug administration reduces pharmacokinetic variations in drug exposure parameters compared to the wide inter-patient variations typically observed following systemic or oral administration. Second, the removal of the solid tumour mass effectively removes any complications caused by microenvironmental conditions. In addition, the comet assay is relatively rapid and from a logistical point of view, it should be feasible to obtain data within the 24 hour window between surgery and chemotherapy. Future studies will determine whether the comet assay or conceptually similar approaches have predictive value in the clinic.

With regards to the second issue, there are similarities between MMC and current approaches used to develop predictive assays for targeted therapies. The most obvious of which is the use of single markers to identify those patients whose tumours are most likely to respond. As described above, the prospects of predicting tumour response to MMC based upon analysis of a single enzyme are poor, which raises the question of whether or not similar mistakes are being made with the new generation of targeted therapies? As in the case of enzyme directed bioreductive drug development, the underlying principle is that if a tumour expresses the target, then that tumour should respond. If this is to be successful, the drug should be specific for its target. Once the drug/target interaction has occurred, downstream events leading to a therapeutic response should be the same within a specific cancer type. Whilst modern target orientated therapies are likely to have a much simpler mechanism of action than MMC, it is generally accepted that the majority of small molecule therapeutics have multiple targets. In the case of Imatinib for example, this compound was designed to inhibit tyrosine kinase activity of the BCR-Abl protein produced by a single genetic event in chronic myelogenous leukaemia. Subsequent studies however demonstrated that imatinib is also active against gastrointestinal stromal tumours that harbour mutations in KIT (112). This illustrates the point that even with targeted drugs, multiple targets and mechanisms of action exist.

Once the drug has bound or interacted with its target, will this ultimately lead to a therapeutic response in all circumstances? The tumour microenvironment is known to influence therapeutic outcome and it is associated with resistance to both radiotherapy and chemotherapy (113, 114). Recent studies have demonstrated that oxygen deprivation results in decreased expression of pro-apoptotic proteins leading to decreased drug induced apoptosis and clonogenic

resistance *in vitro* (115). The tumour microenvironment may therefore have a profound influence on therapeutic outcome, independent of the expression of specific targets in tumour cells. Within this theme, the three dimensional geometry of solid tumours also introduces significant problems related to drug delivery. The poor vascular supply, in conjunction with high interstitial pressures within the tumour, is a recognised barrier to the delivery of therapeutic agents (116, 117). Even if tumour cells express the specific target, there can be no guarantee that sufficient drug will be delivered to cells to elicit a therapeutic response. Whilst significant challenges exist, the use of assays that mimic more closely the heterogeneity within tumours in terms of physiological conditions and drug distribution represent a possible way forward to achieving our goal of individualised therapy.

In conclusion, whilst the modern era of targeted therapeutics offers the potential to tailor therapy to individual patients, past experience with chemotherapeutics such as MMC has demonstrated that prediction of response based on analysis of single target molecules is unlikely to be accurate. Complex mechanisms of action and the effect of the tumour microenvironment on cell biology and drug delivery could thwart attempts to individualise therapies as has been the case with many chemotherapeutic agents. The challenge for the future is to develop assays that mimic key aspects of tumour biology whilst retaining the robustness, simplicity and reliability required for routinely predicting tumour response in a clinical setting. Furthermore, incorporating such assays into pre-clinical and early clinical studies would assist in the more rapid development and evaluation of predictive assays.

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