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Formation of DNA interstrand cross-links as a marker of Mitomycin C bioreductive activation and chemosensitivity

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Abstract

Tumour response to Mitomycin C (MMC) is heterogenous and past attempts to predict clinical response based on enzyme activities have proven unsatisfactory. Using *in vitro* techniques, the aim of this study was to determine if the induction of DNA interstrand cross-links correlated with cellular response and to assess if DNA repair and induction of apoptosis influenced MMC chemosensitivity. Poor correlations were found between sensitivity and both DNA repair and induction of apoptosis suggesting that these processes do not play a major role in determining cellular response to MMC. In contrast, there was good correlation between the induction of DNA interstrand cross-links as determined by the alkaline comet assay and cellular response, suggesting that the biochemical events leading to DNA damage are the key factors that determine cellular response *in vitro*. Further studies are required to assess whether this approach as a mean of prediction has practical applications *in vivo*.

Keywords: Bioreductive drugs; Mitomycin C; Predictive test; DNA damage; Comet assay

1. Introduction

Mitomycin C (MMC) is a quinone based bioreductive drug that is used clinically to treat a variety of malignancies including head and neck cancers and superficial transitional cell carcinoma (TCC) of the bladder [1]. Tumour response is, however, heterogeneous with a broad spectrum of clinical outcome even in patients with histologically identical tumours. In patients with superficial bladder cancers for example, time to first recurrence range from 3 months to over 72 months following intravesical administration of MMC and heterogeneity was observed through grades and within grades [2,3]. There is therefore a need to develop predictive assays that can accurately forecast tumour sensitivity and tailor chemotherapy towards individual patients who are most likely to benefit.

The ability to predict tumour response has been a key objective in the concept of enzyme directed bioreductive drug development [4]. The cornerstones of this concept are the development of compounds that are bioreductively activated by specific reductases (under aerobic and/or hypoxic conditions) and the prediction of response based upon tumour enzymology and/or hypoxia. The metabolic activation of MMC has been extensively studied and several reductases have been implicated [5]. These include two-electron reductases such as NAD(P)H:Quinone oxidoreductase-1 (NQO1), xanthine dehydrogenase and one-electron reductases such as

Abbreviations: TCC, superficial transitional cell carcinoma; MMC, Mitomycin C; NQO1, NAD(P)H:Quinone oxidoreductase-1 (NQO1, E.C. 1.6.99.2); P450R, cytochrome P450 reductase; ICLs, DNA interstrand cross-links; HBSS, Hanks Balance Salt Solution.

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cytochrome P450 reductase (P450R), cytochrome *b*5 reductase and xanthine oxidase, all of which can reduce MMC to DNA damaging species [6–10]. Attempts to predict tumour response to MMC, based upon analysis of these enzymes (particularly NQO1) has, however, proved challenging with conflicting evidence of good and poor correlations reported in the literature [11–14]. This, along with the fact that K_m values for MMC are similar for several reductases [5], suggests that predicting response based upon analysis of single enzymes involved in MMC bioreductive activation is unlikely to be clinically useful.

It is thought that tumour homogenates would incorporate a broad spectrum of reductases present in the tumour at the time of excision and so Cummings *et al.* [5] have suggested that analysis of MMC metabolism may provide a more accurate measure of bioreductive activation and response. A study published previously in which MMC metabolism in two murines tumours with high and low NQO1 activity was studied through active metabolite production. The study showed that both tumours had similar MMC activation rate and was influenced by other cellular enzymes [15]. However, analysis of active metabolite production is complicated by the fact that these metabolites are reactive species that covalently bind to macromolecules, rendering them invisible to standard analytical techniques. In addition, MMC metabolism may not necessarily correlate with response as detoxification pathways (such as glutathione and glutatione *S* transferase [16,17] may compete with this process. Initial results from a study analysing MMC disappearance instead of metabolite formation was encouraging in that MMC sensitive human tumour xenografts tended to metabolise MMC faster than non-responsive tumours [13]. Alternative 'markers' of bioreductive activation that take into account the broad spectrum of reductases and cellular defence mechanisms may provide a better model. Since the end result of MMC bioreductive activation is DNA damage [5,18], quantitative analysis of DNA damage induction (particularly interstrand cross-link (ICL) formation) in intact cells would effectively circumvent the problems outlined above whilst remaining consistent with the overall concept outlined by Cummings *et al.* [5].

The principle objective of this study was to determine the relationship between chemosensitivity and DNA damage (interstrand cross-links) as measured by the comet assay. Whilst the bioreductive activation process is a key issue, it is important to acknowledge that other factors such as DNA repair and cell death pathways (i.e., apoptosis induction) may also play a prominent role in determining cellular response to MMC. These 'downstream' events have not been studied extensively in the context of predictive assay development for MMC. A secondary objective of this study is to determine the potential impact of downstream events such as DNA repair and apoptosis on cellular response to MMC.

2. Materials and methods

2.1. Cell culture

Cell lines were obtained from the American Tissue Culture Collection (Maryland, USA). H460 and H596 non-small cell lung cancer cells, BE and HT29 colon cancer cells, RT112 and EJ138 bladder cancer cells were grown in RPMI 1640 medium with 10% foetal calf serum, 5 mM L-glutamine, 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The RT4 bladder cancer cells were grown in McCoy's medium with 10% foetal calf serum, 5 mM L-glutamine, 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The T47D breast cancer cells were grown in DMEM medium with 10% foetal calf serum, 5 mM L-glutamine, 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Chemosensitivity

In vitro chemosensitivity to MMC was determined using the MTT assay 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, details of which have been described elsewhere [19]. Briefly, cells were plated into 96 well culture plates at 1×10^3 cells per well (200 μl media per well) and incubated overnight at 37 °C. The following day, media was carefully removed and replaced with fresh media containing MMC. Cells were exposed to a range of MMC concentrations for 1 and 3 h after which they were washed twice with Hanks Balance Salt Solution (HBSS) prior to addition of media. Following 4–6 days (depending on the cell line) incubation at 37 °C, cell survival was determined using the MTT assay. Results are presented as $C \times T$ (where C = MMC concentration (μM) and T = duration of drug exposure (h)) versus % cell survival compared to untreated control cells. IC_{50} values (concentration required to reduce cell survival by 50%) are expressed as the means ± standard deviation of three independent experiments.

2.3. Apoptosis induction

H460 and RT4 cells in mid exponential growth were exposed to MMC for 1 h. At various time intervals after drug exposure, apoptotic cells were identified by Annexin V-FITC/PI dual staining (Calbiochem) and Hoescht 33342 staining (Sigma). Each control and treated cell sample were split into two aliquots. The first aliquot was stained with both Annexin V-FITC and PI. Samples