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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

were analysed by flow cytometry immediately after staining using a FACS Calibur (Becton Dickinson), with 10 000 cells counted in each sample. Cells from the second aliquot were put on slides using a cytospin (Cytospin 4, Thermo Shandon). Slides were fixed in methanol at −20 °C for 30 min, air-dried and rehydrated in PBS for 5 min and then covered with Hoechst 33342 stain (1 μg/ml in dH_2O) and incubated for 15 min in the dark. After washing with PBS, slides were mounted with coverslips using VectaShield fluorescence mounting (Vector Therapeutics) and sealed. Samples were analysed using a fluorescent microscope (Leica), linked to a CCD camera. Live and dying cells were counted from five different fields on each slide. In both cases, percentage of cell death induction was obtained as followed: % cell death induction = % positive staining (treated population) − % positive staining (control population). Each experiment was performed in triplicate.

2.4. DNA cross-links measurements

The alkaline comet assay was used to analyse interstrand DNA cross-link induction by MMC in cancer cells. Exponentially growing cells were treated with MMC for 1 and 3 h. Cells were then washed and fresh medium was added. At various time points between 0 and 6 h after drug exposure, cells were harvested in ice cold PBS by scraping and centrifuged at 2000*g* for 5 min at 4 °C and the pellet resuspended in ice-cold PBS. Cells were incubated with 100 μM hydrogen peroxide to induce strand breaks (maximum induction of strand breaks occurs at this concentration) for 20 min at 4 °C, centrifuged at 2000*g* for 5 min at 4 °C and resuspended in PBS. The comet assay was performed as described elsewhere [20]. Briefly, cell samples were added to an equal volume of 1% low melting point agarose and embedded on a slide coated with 1% normal melting point agarose. When the agarose layer had solidified, another layer of 0.5% low melting point agarose was added and left to solidify. The slides were then incubated in lysis solution (2.5 N NaCl, 10 mM Tris, 100 mM EDTA, pH 10.5, 1% v/v Triton X-100, 1% v/v DMSO) overnight at 4 °C in the dark. Slides were equilibrated in fresh electrophoresis buffer (50 mM NaOH, 1 mM EDTA, 10% v/v DMSO, pH 12.5) and electrophoresed for 30 min at 0.6 V/cm in the dark. Following electrophoresis, slides were washed twice (5 min each) in neutralising buffer (500 mM Tris, pH 7.5) and 5 min in distilled water and dried overnight. Next day, they were rehydrated 30 min with distilled water and stained 30 min with SYBr Gold (Molecular Probe, UK) at a 1:10 000 dilution. The slides were finally illuminated with green light (590 nm filter) under fluorescence microscope (Leica), linked to a CCD camera. DNA migration was analysed using the Comet Assay III software (Perceptive Instruments), by measuring 50 cells per slides. The percentage of DNA cross-linked was obtained as follows: % of DNA cross-linked = 100 − (100 × Tail Extent Moment ($MMC + H_2O_2$) treated cells)/Tail Extent Moment (H_2O_2) treated cells). The percentage of DNA repaired damage was expressed as 100 − (100 × %DNA cross-linked (time 6 h)/%DNA cross-linked (time 0 h)). Each experiment was performed in three independent replicates.

3. Results

3.1. MMC cytotoxicity in vitro

The response of a panel of human tumour cell lines to MMC was determined *in vitro* and IC_{50} values following both a 1 and 3 h exposure are presented in Fig. 1A. Marked differences were seen in response to the drug, depending on the duration of exposure. Following a 1 h drug exposure a broad spectrum of response was obtained with IC_{50} values ranging from 0.77 ± 0.28 to 117.87 ± 57.45 μM (Fig. 1A). In contrast, the response of cell lines following a 3 h exposure to MMC was more uniform with IC_{50} values ranging from 0.63 ± 0.23 to 1.76 ± 0.78 μM. The exception was H596 cells, which retained a high IC_{50} (42.50 ± 1.00 μM). Particularly marked differences in sensitivity to MMC between 1 and 3 h exposure were seen in RT4 cells, with IC_{50}

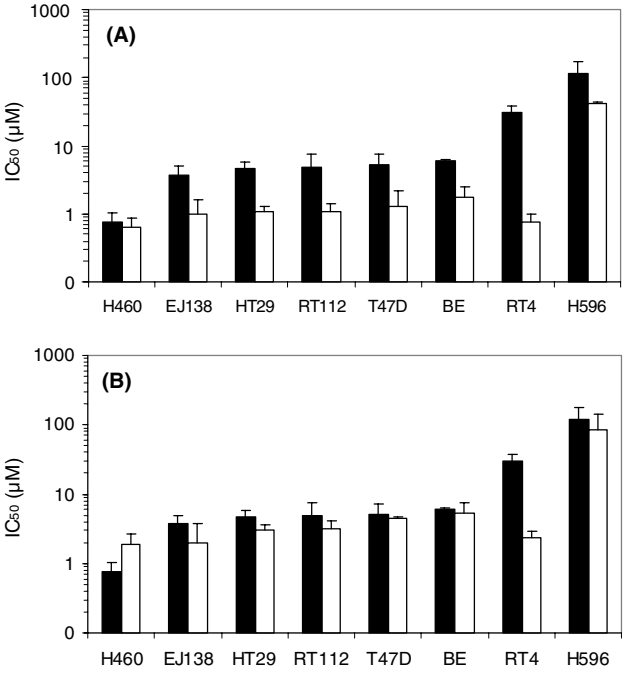

Fig. 1. MMC cytotoxicity in various human cancer cell lines obtained after 1 h (■) and 3 h (□) exposure. (A) Cytotoxicity as IC_{50} values. (B) Cytotoxicity as $C \times T$ values. Each value presented represents the means ± standard deviation from three independent experiments.

values decreasing from 30.20 ± 7.33 to 0.77 ± 0.20 μM, respectively (Fig. 1A). Sensitivity to a cytotoxic agent can also be expressed as a factor reflecting concentration and time (i.e., μM h). When expressed in these terms, dose response curves and IC_{50} values are similar for the majority of cell lines (Fig. 1B). A notable exception again is the RT4 cell line where the IC_{50} values range from 30.20 ± 7.33 μM h (at 1 h exposure) and 2.33 ± 0.58 μM h (at 3 h exposure). In this case, cells killed is proportional to T rather than the product of $C \times T$. In contrast, H460 cells are relatively more resistant to MMC following 3 h exposure (IC_{50} = 1.85 ± 0.84 μM h) compared to response following a 1 h (IC_{50} = 0.77 ± 0.28 μM h) although the magnitude of the effect is reduced compared to RT4 cells. The results demonstrate that duration of drug exposure is an important parameter influencing sensitivity to MMC.

3.2. MMC apoptosis induction

Annexin V-FITC/PI dual staining and Hoechst staining were used to measure apoptosis induction in H460 (MMC sensitive) and RT4 (MMC resistant) cell lines. RT4 cells were chosen over H596 cells due reduced doubling time (data not shown). Etoposide (20 μM) was used as a positive control. After 48 h exposure to etoposide, flow cytometry analysis showed the induction of cell death in 62.9 ± 14.3% of H460 cells (Fig. 2A) and 19.7 ± 9.1% in RT4 cells (Fig. 2B). After exposure to 5 μM MMC for 1 h, there was a maximum of 11.8 ± 4.6% cell death in H460 cells at 24 h and 15.6 ± 10.3% at 96 h and RT4 cell, respectively. Results obtained by Hoechst staining confirmed this finding as illustrated in Fig. 2C–H. Hoechst 33342 is a fluorescent stain that binds to DNA and allows the observation of nuclei under a fluorescence microscope. Normal cell

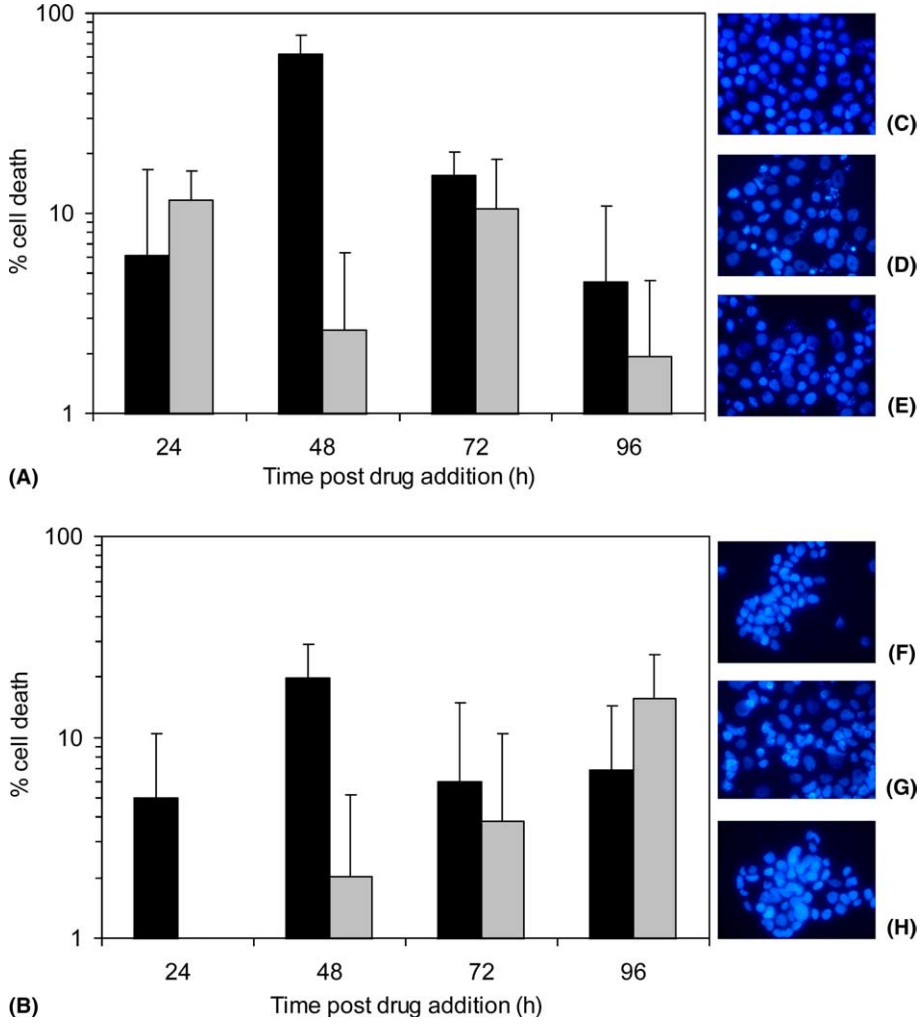

Fig. 2. Induction of cell death in cell lines. Flow cytometry analysis of cell death induction in H460 (panel A) and RT4 cells (panel B) following exposure to either MMC (1 h at 5 μM, grey bars) or etoposide (continuous exposure at 20 μM). Each value represents the means ± standard deviation for three independent experiments. Panel C (control), D (etoposide), E (MMC) illustrate induction of cell death in H460 cells measured by Hoechst staining. Panel F (control), G (etoposide), H (MMC) illustrate induction of cell death in RT4 cells measured by Hoechst staining.

nuclei appear round and homogeneously stained whereas apoptotic cells are revealed by the presence of smaller, brighter, granular structures which correspond to apoptotic bodies resulting from nuclear condensation. These worked examples showed the absence of apoptotic cells in H460 and RT4 untreated cells (Figs. 2C and F), a strong apoptosis induction in H460 and RT4 cells treated with etoposide (20 μM) for 48 h (Figs. 2D and G) and only low apoptosis induction in H460 cells 48 h after treatment with 5 μM MMC (1 h exposure, Fig. 2E). No apoptotic cell was observed in RT4 cell population treated with MMC (5 μM, 1 h) and left to recover 48 h (Fig. 2H). These results indicated that maximum cell death induction following exposure to MMC was similar in both cell lines despite the wide variation in sensitivity to MMC.

3.3. DNA damage induction and repair study

The alkaline comet assay was used to measure interstrand DNA cross-link formation after MMC treatment in human cancer cell lines. Six cell lines, selected from the initial panel to represent the full range of sensitivity, were exposed for 1 and 3 h to a relevant therapeutic dose of 5 μM of MMC. The assay was performed immediately after drug exposure and 3 presents the results obtained. After 1 h exposure, 70.7 ± 12.2% of DNA was cross-linked in H460 cells (the cell line most sensitive to MMC), compared to none and only 4.0 ± 3.5% of DNA cross-linked in RT4 and H596 cells, respectively (the two most resistant cell lines to MMC). All the cell lines had increased DNA cross-link formation when drug exposure was extended to 3 h. In the H460 cell line, cross-linking increased to 84.8 ± 13.5% with longer exposure and to 59.4 ± 20.7% and 27.6 ± 7.8% in RT4 and H596 cells, respectively. All cell lines showed the same pattern with increased DNA damage with longer exposure time to MMC.

In order to further characterise DNA cross-link formation, a time course study was undertaken to follow the formation of ICLs through time in both H460 and RT4 cells (Figs. 4A and B). Fig. 4A presents the results obtained with H460 cells. The extent of DNA cross-linked decreased down to 33.6 ± 16.8% after 24 h recovery. After exposing RT4 cells to MMC (5 μM) for an hour and leaving them to recover for 24 h, DNA cross-linking could be detected 3 h after the drug exposure and increased up to 32.97 ± 1.89% of DNA cross-linked by 6 h (Fig. 4B). After 24 h of recovery, DNA was repaired and only 21.0 ± 15.7% of the RT4 cell DNA remained cross-linked, indicating that approximately 36% of cross-linked DNA was repaired. Fig. 4C presents the results of repair time-course experiments in H460, HT29 and BE cells. H460 cells had repaired approximately 30% of the cross-linked DNA after 6 h recovery, whereas BE cells, which are less sensitive to MMC, repaired all of the damage induced. HT29 cells, which have a similar IC_{50} to BE cells, could only repair around 10.3% of the DNA damage.

The ability of these three cell lines to repair ICLs did not to correlate with their sensitivity to MMC. The relationship between ICL formation and chemosensitivity is presented in Fig. 5. A good correlation was established between these two parameters ($r^2 = 0.66$) with increased sensitivity associated with increased ICL formation.

4. Discussion

The ability to tailor chemotherapy to individual patients is an integral part of bioreductive drug development. However, despite extensive studies on the mechanism of action of MMC, predictive assay development based on tumour enzymology has generated inconsistent and conflicting results in both experimental and clinical settings [3,11–14]. Previously published studies have shown that BE and H596 cells have very low activity level (<0.1 nmol/mg/min) but they present different level of sensitivity to MMC with IC_{50} values of 6.02 ± 0.36 and 117.87 ± 57.45 μM, respectively (Fig. 1) [21,22]. On the other hand, BE and HT29 cells have similar IC_{50} values (6.02 ± 0.36 and 4.60 ± 1.23 μM) but HT29 cells were shown to have a high NQO1 activity (688 ± 52 nmol/mg/min) [22]. These data support the idea that MMC activation is complex, involving several reductases and other proteins [5,23,24] together with many physiological factors (e.g., pH and hypoxia) modulating activity of MMC [25,26], and evades accurate measurement leading to poor predictive value of assays based upon analysis of individual reductases. It is also possible that biochemical events post MMC activation may also play a prominent role in determining response. MMC is known to induce apoptosis in cell lines [27,28] and apoptosis measurements have been suggested as a potential predictive marker of response to MMC chemotherapy [29]. In this study, although low levels of apoptosis induction were observed (Fig. 2), there was no indication of a close relationship between apoptosis induction and response in either MMC sensitive (H460) or resistant (RT4) cells lines. These results support those of Gontero and colleagues [30] that showed no correlation between caspase-3 levels and resistance to MMC, suggesting that non-apoptotic modes of cell death [31] predominate in MMC therapy.

With regards to DNA repair kinetics, marked differences exist in the ability of 4 cell lines to repair MMC induced ICL (Fig. 4C). For example, BE cells showed almost complete repair of DNA damage 6 h after drug exposure, whereas H460 and HT-29 cells showed moderate or poor repair of ICLs, respectively. RT4 cells were quite distinct from the other cell lines in that no ICL formation was observed immediately after a 1 h drug

exposure (Fig. 3) but significant levels of ICL formation was observed after a 6 h recovery period (Fig. 4B). A more detailed time course analysis of ICL formation in RT4 cells following a 1 h drug exposure is presented in Fig. 4B and this demonstrated a time-dependent increase in ICL formation. A mechanistic explanation for this observation is not known but it illustrates the fact that inherent differences in both ICL formation and DNA repair exist between different cell lines. In terms of the relationship between DNA repair kinetics and chemosensitivity, the repair proficient BE cell line and the repair deficient HT-29 cell line have similar IC_{50} values following a 1 h drug exposure (4.60 ± 1.23 and 6.02 ± 0.36 μM, respectively). Whilst there are marked differences in repair capacity, these preliminary results (in conjunction with the unusual pattern of ICL formation in RT4 cells) suggest that analysis of DNA repair is unlikely to be of value in terms of developing a predictive assay for MMC.

This study has refined the concept initially proposed by Cummings *et al.* [5] and analysed the induction of DNA ICLs as a marker of the bioreductive activation process and compared this with chemosensitivity *in vitro*. In all cases, exposure to MMC for 3 h resulted in increased ICL formation although notable differences in ICL formation exist both between individual cell lines and the duration of drug exposure. This is particularly marked in the case of RT4 cells where no ICL formation was observed after a 1 h exposure but high ICL levels were produced after a 3 h exposure. This correlates with both the marked increase in sensitivity of RT4 cells following a 3 h drug exposure (Fig. 1) and consequently the unusual relationship between $C \times T$ and response (where cell kill is proportional to T rather than $C \times T$ as in the majority of other cell lines). The relationship between ICLs formation and cell line sensitivity was first suggested by O'Connor and Kohn [32], who showed a correlation between ICLs formation measured by DNA alkaline elution and L1210 murine cells sensitivity to 4 different nitrogen mustards. These data were confirmed later in a study demonstrating a correlation between IC_{50} values for 3 aromatic nitrogen mustards and the extent of ICLs determined by alkaline elution assay in two

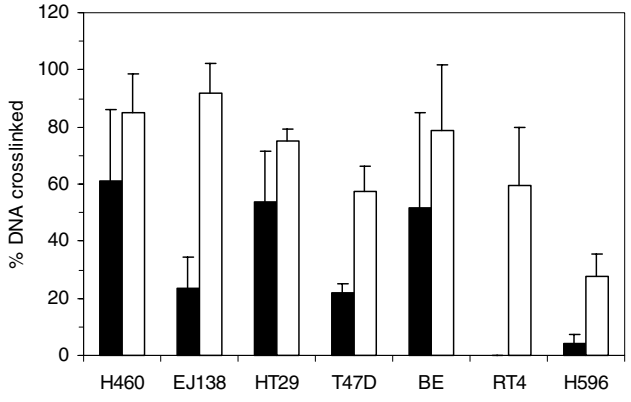

Fig. 3. DNA interstrand cross-links induction and repair kinetics. Representation of the induction of DNA interstrand cross-links in cancer cell lines exposed to MMC (5 μM) for 1 h (■) and 3 h (□).

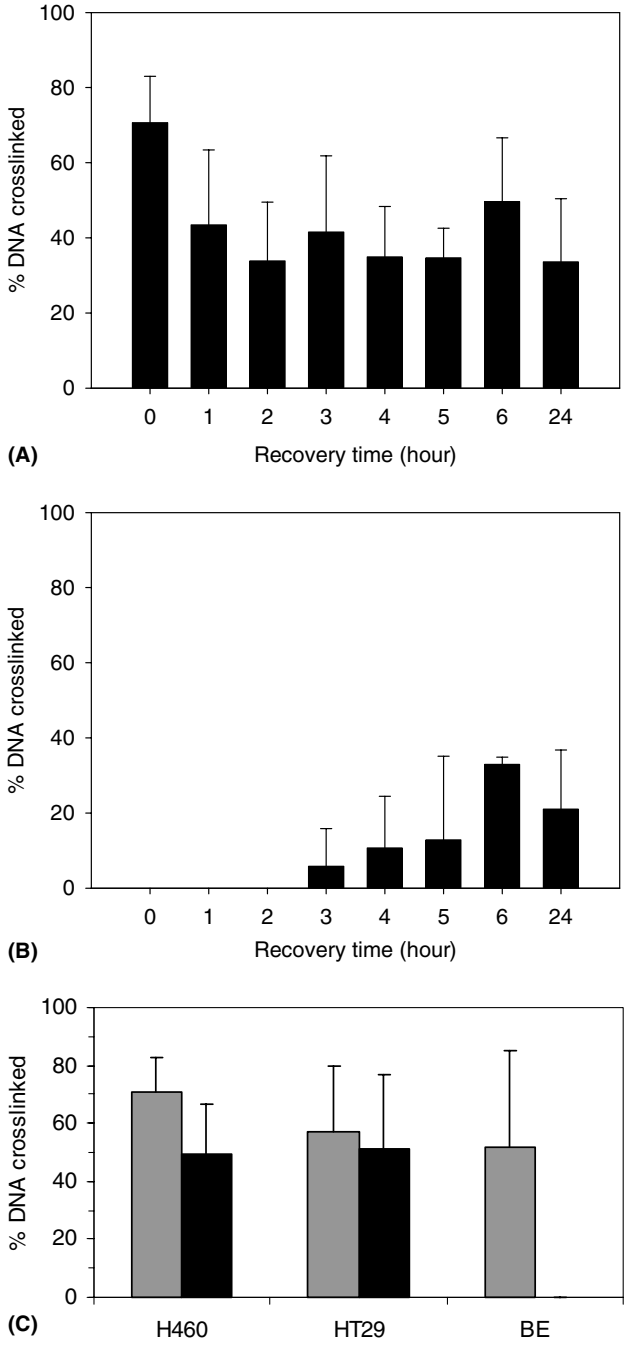

Fig. 4. Panel A represents the kinetic of cross-links formation in H460 cells exposed to MMC (5 μM) for 1 h and left to recover over 24 h. Panel B represents the kinetic of cross-links formation in RT4 cells exposed to MMC (5 μM) for 1 h and left to recover over 24 h. Panel C represents the extent of cross-links measured in cells exposed to MMC (5 μM) for 1 h (▒) and left to recover for 6 h (■). Each value presented represents the means ± standard deviation for three independent experiments.

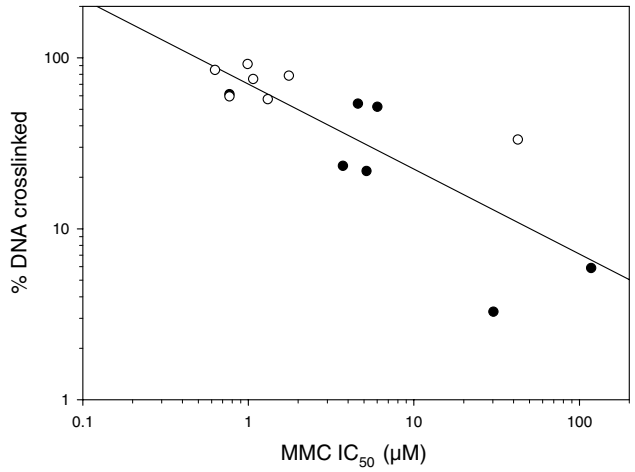

Fig. 5. Relationship between extent of DNA interstrand cross-link formation and chemosensitivity to MMC *in vitro* after 1 h (•) and 3 h (○) exposure.

human cancer cell lines [33]. Together, these data indicate that ICLs formation is a good indicator of alkylating agent biological activity. Nevertheless, it should be acknowledged that there is still variability within the data set and further improvements are required before *in vivo* studies can be conducted. For example, MMC induces other forms of DNA damage (i.e., mono-adducts at the N7 and N2 position of guanine [5]) so whilst ICLs are generally regarded as the most toxic lesion, other forms of DNA damage may still contribute towards cytotoxicity and cannot be ignored.

In conclusion, this study demonstrates that analysis of DNA ICL formation following exposure to MMC provides a good indicator of MMC chemosensitivity. This result, in conjunction with the lack of evidence supporting a strong role for repair or apoptosis in MMC sensitivity, suggests that the bioreductive activation process remains the major determinant of cellular response to MMC *in vitro*. The comet assay has the advantage of using intact cells that will express a variety of proteins/reductases involved in the bioreductive activation process. In addition, detoxification pathways and other cellular defence mechanisms are also indirectly assessed as ICL formation is likely to represent the balance between bioactivation on the one hand and detoxification processes on the other. Whilst predicting response to therapy based on analysis of single enzymes remains an attractive proposition, it is probably too simplistic for MMC and cellular based procedures such as the comet assay represent a potential way forward. Recent studies have demonstrated that the comet assay provides a good indication of response to radiotherapy [34,35] and is simple, inexpensive, rapid and requires small biopsy samples [34]. Analysis of DNA damage caused by MMC using the comet assay may provide a good indication of response and further studies to address this issue are warranted.

Conflict of interest statement

None declared.

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