Detection of Mitomycin C-DNA Adducts in Vivo by 32P-Postlabeling: Time Course for Formation and Removal of Adducts and Biochemical Modulation

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ABSTRACT

Mitomycin C (MMC) is a DNA cross-linking agent that has been used in cancer chemotherapy for over 20 years, yet little is known either qualitatively or quantitatively about MMC-induced DNA adduct formation and repair in vivo. As an initial means of investigating this, we used a recently developed 32P-postlabeling assay to examine the formation and loss of MMC-DNA adducts in the tissues of a simple in vivo model system, the chick embryo, following treatment with a chemotherapeutic dose of MMC. As early as 15 min after MMC treatment, four adducts could be detected in the liver which were tentatively identified as the (CpG) N2G-MMC-N2G interstrand cross-link, the bifunctionally activated MMC-N2G monoaadduct, and two isomers (α and β) of the monofunctionally activated MMC-N2G monoadduct. The (CpG) N2G-MMC-N2G interstrand cross-link appears to be a poor substrate for nucleases P1 and/or T4 kinase and was not evaluable by this assay. Levels of all four detectable adducts increased substantially within the first 2 h after MMC treatment, reached maximal levels by 6 h, and decreased progressively thereafter through 24 h, although low levels of certain adducts persisted beyond 24 h. Lung and kidney had comparable levels of total MMC adducts, which were approximately 60% those of the liver, and there were no significant differences in the proportion of specific adducts among the three tissues. The interstrand cross-link represented ~13-14% of the total MMC adducts, which is approximately 5-fold greater than the proportion of CpG sites in the genome. In addition, the interstrand cross-link was selectively decreased after 16 h relative to the three monoaadducts, suggesting preferential repair. The effect of modulating different components of the Phase I and Phase II drug metabolism on MMC adduct formation, using either glutathione, 3,4,3′,4′-tetrachlorobiphenyl, dexamethasone, buthionine sulfoximine, ethacrynic acid, or N-acetylcysteine pretreatments, was examined to characterize the possible pathways of MMC metabolism and adduct formation in vivo. Surprisingly, none of these pretreatments had a significant effect on individual or total adducts with the exception of dexamethasone, which caused an almost 2-fold proportional increase in all four adducts in the liver.

INTRODUCTION

MMC is a bifunctional cross-linking agent that requires sequential chemical or enzymatic reductions to form covalent adducts with DNA principally at the N⁰ position of guanine (G), forming monofunctionally and bifunctionally activated G-MMC monoadducts, and G-MMC-G interstrand and intrastrand cross-links at CpG and GpG sites, respectively (1-3). Although a great deal is now known about the chemistry of MMC adduction to DNA in vitro and in cell culture, very little is known about MMC effects in intact animals and humans. In addition, although MMC has been used clinically in cancer chemotherapy for over 20 years, there is still little known about the pharmacokinetics or pharmacodynamics of MMC-DNA adduction in humans. It is generally believed that the interstrand cross-link is principally responsible for the antitumor activity of MMC, because these lesions are lethal if unrepaired (4, 5). However, correlations between specific MMC-DNA adducts and specific biological end points, such as alterations in DNA replication, RNA transcription, and cytotoxicity, have yet to be determined. Our laboratory has previously provided evidence that MMC and other genotoxic agents appear to preferentially "target" their effects to specific genes in vivo (6-8). We were, therefore, interested in whether the potent effects of MMC on gene expression could be correlated with specific types of MMC-induced DNA lesions in vivo.

A highly sensitive method is required to detect and quantify MMC-DNA lesions at the low levels that occur following physiologically relevant doses. 32P-Postlabeling is generally the most sensitive method for the detection of DNA adducts and is capable of detecting one adduct per 10⁷ to 10¹⁰ normal nucleotides (i.e., as little as one adduct per cell; reviewed in Refs. 9 and 10). The 32P-postlabeling assay has now been used by over 100 laboratories to detect a wide variety of specific DNA adducts resulting from chemical exposures related to occupational settings, environmental contamination, diet, and clinical drug use (10). Recently, a 32P-postlabeling procedure was developed for detecting the major MMC monofunctional monoadduct, N²-(2′,7′-diaminominotheno-1′-α-yl)-2′-deoxyguanylic acid, which also had the potential for detecting other MMC-DNA adducts (11). Using this methodology, we examined whether specific MMC-DNA adducts could be detected in various tissues following in vivo treatment of a simple whole-animal model system, the chick embryo, with pharmaco logically relevant but nonovertly toxic doses of MMC. Subsequently, the time course for the formation and removal of specific MMC-DNA adducts was examined to determine whether there was a temporal relationship between specific adduct levels and previously observed MMC-induced alterations in gene expression. Finally, we examined the effects of various biochemical modulators of Phase I and II activation and detoxification pathways to determine whether alterations in specific pathways would alter MMC metabolism or MMC-DNA adduct formation in vivo.

MATERIALS AND METHODS

Preparation of MMC-modified DNA Standards. A duplex 23-bp oligonucleotide containing a single CpG site (referred to as 3′/8, top strand sequence AT/3AA/4TACGTTATTTAATTTAAAA) was reacted with MMC in the presence of SDT (6.0 mM Na₂SO₄, 1.5 mM MMC:1.5 mM nucleotides) at 4°C under argon essentially as described previously (12). The interstrand cross-link was subsequently purified from the unreacted duplex using a high temperature, high resolution Sephacryl S-100 chromatography method, and characterized as...
described (12). Salmon sperm DNA (43% GC content) was reacted with MMC under similar reducing conditions, and free drug was removed by ethanol precipitation. An 87-bp DNA fragment (59.8% GC content) generated by PCR under similar reducing conditions, and free drug was removed by ethanol precipitation (12).

**Treatment of Chick Embryos and DNA Isolation.** Fertile White Leghorn chicken eggs (Truslow Farms, Inc., Chestertown, MD) were incubated as described previously (8, 14). Chick embryos at the appropriate DI were administered a single dose of 30 μmol/kg MMC (100 μg/egg at 14 DI and 125 μg at 15 DI; Ref. 8) in 100 μl of water. For experiments using biochemical modulators, chick embryos were administered either glucethimide (0.5 mmol/kg; Ref. 15), dexamethasone-21-phosphate (2.5 mmol/kg; Ref. 7), TCB (2.5 mmol/kg; Ref. 14), or BSO (1.5 mmol/kg; Ref. 16) 24 h prior to treatment with MMC (15 μmol/kg). NAC (1.5 mmol/kg) and EA (200 μmol/kg) were administered 2.5 and 1 h prior to MMC (15 μmol/kg), respectively. At the appropriate time point, livers were removed; one lobe from each chick embryo was used for postlabeling, whereas the other lobe was reserved for DTD and GSH assays.

**32P-Postlabeling Assay for MMC-DNA Adducts.** For postlabeling, the liver lobe was homogenized by repeated passage through an 18-gauge needle, and DNA was isolated using a genomic isolation kit (Qiagen). DNA was resuspended in water and stored at —70°C. Typical DNA yields were 200–400 μg of DNA per lobe of liver with an A260/A280 ratio of 1.7–1.8. Samples were routinely monitored by HPLC (as described below) to assess RNA contamination. A 2.5-μg aliquot of DNA was dried down in a Speed Vac (Savant) and digested with NP1 and PAP (80 mM sodium acetate [pH 5.0], 150 μM ZnCl2, 4 μg of NP1, and 200 milliunits of PAP in a total of 10 μl; 1 h at 37°C) as described (11). The digestion was terminated by adjusting the pH to 11.5 with 2.5 μl of 0.5 M Tris base, and an aliquot was subjected to HPLC analysis for determination of total nucleotide content. The remainder of the sample was used for 32P-postlabeling essentially as described previously (11). Briefly, the specific activity of each lot of [γ-32P]ATP was calculated by radiolabeling deoxyadenosine 3'-monophosphate followed by TLC in 0.3 M ammonium sulfate buffer with 10 mM sodium phosphate (pH 7.5). Following 5'-end labeling with [γ-32P]ATP and T4 kinase and subsequent treatment with apyrase (20 milliunits/μl in 20 mM bicine), the 32P-labeled adducts were purified using a C18 solid-phase extraction column (SepPak Vac; Waters; Ref. 11). The 32P-labeled adducts were dried and subsequently further digested with SVPD as described (11). Radiolabeled adducts were separated by two-dimensional TLC on polyethyleneimine-cellulose plates (MACHEREY-NAGEL) using 1 m NaH2PO4, pH 6.5 (D1), and 1.3 lithium formate/2.1 M urea, pH 3.5 (D1). Adduct signals were visualized by autoradiography, and these regions were cut from TLC plates and quantified by liquid scintillation. Adduct levels were calculated based on the radioactivity of each spot, the specific activity of the ATP used in the kinase reaction, and the nucleotide content based on the HPLC analysis (11). Values obtained for the interstrand cross-link were divided by 2 to account for 32P-endlabeling of both OH groups of this adduct fragment.4 Statistical analyses of data were performed by ANOVA and Student's t test using the InStat software program (version 2.0; Graphpad Software).

**HPLC Analysis.** An aliquot from each NP1/PAP-digested sample was analyzed for total nucleoside content by HPLC (Hewlett Packard 1090 or 1050; C18 column, Rainin Microsorb-MV, 5 μm, 4.6 mm × 25 cm; flow rate, 1 ml/min). The following gradient program was used to develop the chromatogram: 100% buffer A (50 mM KH2PO4, pH 4.0, 2.5% methanol) for 3 min to 100% buffer B (50 mM KH2PO4, pH 4.0, 20% methanol) over 9 min; isocratic with a 10-min hold at 100% buffer B for 10 min; gradient from 100% buffer B to 60% buffer B over 4.2 min; then gradient to 100% buffer A over 0.8 min, followed by a 10-min wash/equilibration in 100% buffer A. A standard nucleoside mix was used to calculate the total nucleoside content of each sample (11). 

**Analysis of DTD and GSH Levels.** Each liver lobe was homogenized in 0.25 M sucrose; one-half was reserved for GSH determination, whereas the other half was used for DTD activity. The cytosolic fraction was isolated and assayed for GSH content using a fluorimetric assay as described (17). For DTD assays, cytosolic and microsomal fractions were prepared, and the cytosol was assayed for DTD enzyme activity as described (18). Values were normalized for total protein content as determined by the BCA assay (Pierce, Rockford, IL).

**RESULTS**

The general scheme for postlabeling MMC-DNA adducts is shown in Fig. 1. The 3'-phosphodiester bond at an added guanine is NP1 resistant, making this an ideal method for adduct enrichment (9, 10).

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4 A. E. Maccubbin, unpublished results.
of these latter two adducts has not been performed with purified standards. However, alterations in the relative proportion of the different spots was shown to be dependent on the method of activation of MMC, further supporting these assignments. Under monofunctional activation conditions (Fig. 4), spots 3 and 4 were observed mainly, whereas under bifunctional activation conditions (Fig. 3), spots 1 and 2 were observed mainly, consistent with their previous characterization (1, 11, 13, 19–21). $^{32}$P-Postlabeling of an oligonucleotide containing a single GpG site under conditions favoring formation of the N2G-MMC-N2G intrastrand cross-link yielded ambig-

NP1 treatment results in unmodified nucleosides (N), monoadducted nucleotides ($3^pN$X, where X indicates an adduct), and cross-linked nucleotides ($3^pN-GxpN$). Because of the requirement for a 3'-phosphate for efficient 5'-end labeling by T4 kinase, the adducted nucleotides were preferentially radiolabeled. Subsequent treatment with SVPD yields 5'-end labeled adducted nucleosides ($3^pGx$, $3^pGx-xG32p$; reviewed in Refs. 9 and 10). To allow identification of specific MMC-DNA adducts, various authentic standards were synthesized. The monofunctional monoadduct, N2-(2'ß,7'-diaminomitosen-1'-a-yl)-2'-deoxyguanylic acid, which is the major MMC monoadduct (a isomer), was synthesized using reaction conditions (H2/Pt,0) that favor monofunctional activation conditions (19). $^{32}$P-Postlabeling of this standard had been shown previously to produce one adduct spot (referred to as “spot 3”; Ref. 11). $^{32}$P-Postlabeling of the highly purified and previously characterized (12) MMC-DNA interstrand cross-link, N~-(2'ß,7'-diamino-10'-deoxyguanyl-N2-yl-mitosen-1'-a-yl)-2'-deoxyguanylic acid, generated in vitro produced two spots (Fig. 2, referred to as “spot 1” and “spot 5”). Greater than 97% of the total radioactivity was present in spot 1, which represents the (CpG) N2-G-MMC-N2-G cross-link. Spot 5 was never observed in in vivo-treated samples and, therefore, is not believed to be biologically relevant. Spot 5 varied in intensity, depending on how long the cross-link was stored prior to analysis, and most likely represents a major degradation product or side product, perhaps involving oxidative damage produced from the SDT reducing conditions.

Salmon sperm DNA was reacted in vitro with MMC under optimal bifunctional reducing conditions and $^{32}$P-postlabeled, which produced six spots (Fig. 3). In addition to spots 1, 3, and 5, two other spots (“spot 4” and “spot 2”) were observed, which most likely represent the $\beta$-isomer of the monofunctional monoadduct, N2-(2'ß,7'-diaminomitosen-1'-a-yl)-2'-deoxyguanylic acid, and the bifunctional monoadduct, N2-(10'-decarbamoyl-2',7'-diaminomitosen-1'-a-yl)-2'-deoxyguanylic acid, respectively (13, 20), although absolute confirmation of these latter two adducts has not been performed with purified standards. However, alterations in the relative proportion of the different spots was shown to be dependent on the method of activation of MMC, further supporting these assignments. Under monofunctional activation conditions (Fig. 4), spots 3 and 4 were observed mainly, whereas under bifunctional activation conditions (Fig. 3), spots 1 and 2 were observed mainly, consistent with their previous characterization (1, 11, 13, 19–21). $^{32}$P-Postlabeling of an oligonucleotide containing a single GpG site under conditions favoring formation of the N2G-MMC-N2G intrastrand cross-link yielded ambig-

Fig. 2. $^{32}$P-Postlabeling of the MMC-DNA interstrand cross-link produced in vitro. A 23-bp duplex oligonucleotide containing a central CpG N2G-MMC-N2G interstrand cross-link was synthesized and purified to homogeneity (12) and subjected to $^{32}$P-postlabeling (see Fig. 1 and “Materials and Methods”). Two adducts were observed that are referred to as spots 1 and 5 in the text. Spot 1 corresponds to the interstrand cross-link. Spot 5 is unidentified but is likely either another adduct produced from the in vitro SDT reaction conditions and/or a breakdown product of the cross-link.

Fig. 3. $^{32}$P-Postlabeling of salmon sperm DNA treated with MMC under bifunctional reducing conditions in vitro. Purified salmon sperm DNA was treated with MMC under bifunctional reducing conditions in vitro (see “Materials and Methods”) and subjected to $^{32}$P-postlabeling (see Fig. 1 and “Materials and Methods”). Five adducts were detected and are referred to as spots 1 through 5. Spot 1 represents the interstrand cross-link, and spot 5 is an artifact of the in vitro reaction (see Fig. 2). Spot 3 represents the major monofunctional monoadduct (a isomer) as described previously (11). Spots 2 and 4 represent the bifunctional monoadduct and the $\beta$-isomer of the monofunctional monoadduct, respectively. Spots 3 and 4 are not visible on this exposure and required longer autoradiograph times for detection in these samples (data not shown; see Table 1). Their positions are shown by the arrows.

Fig. 4. $^{32}$P-Postlabeling of an 87-bp synthetic duplex treated with MMC under monofunctional alkylating conditions in vitro. An 87-bp duplex DNA fragment (60% GC content) was treated with xanthine oxidase/NADH under argon as described in “Materials and Methods” and subjected to $^{32}$P-postlabeling (see Fig. 1 and “Materials and Methods”). Five adducts were detected and are referred to as spots 1 through 5. Adducts 1–5 were detected as described in Figs. 2 and 3 above, and an additional unidentified adduct (spot 5) was also detected in these samples.
uous results (data not shown), suggesting that this adduct is not a good substrate in the kinase reaction. Thus, a different method may be necessary to assess this adduct by $^{32}$P-postlabeling. Validation of the quantitative nature of the assay has been described previously (11). In addition, radiolabeled [H]porfiromycin was used as an indicator of MMC binding, and similar total adducts/nucleotides were obtained by the two methods (data not shown). Several of the same MMC-treated samples were independently $^{32}$P-postlabeled and analyzed in two different laboratories (A. E. M., Roswell Park, and J. W. H., Dartmouth) to examine interlaboratory variability, and comparable results were obtained (data not shown).

We then examined whether MMC-DNA adducts could be detected in vivo at physiologically relevant doses. The chick embryo was chosen as a simple and useful in vivo model system for these studies. Embryos were treated with a single dose of 30 μmol/kg MMC (~LD$_3$), which had previously been shown to cause little or no overt cytotoxicity over a 96-h period, but which caused significant suppression of DNA and RNA synthesis, and which also caused profound changes in expression of specific genes (8). $^5$DNA was isolated from the liver at various times after MMC treatment and analyzed by $^{32}$P-postlabeling. Representative autoradiographs of both solvent- and MMC-treated embryos are shown in Fig. 5. Four spots were detected corresponding to the interstrand cross-link (spot 1), the bifunctional monoadduct (spot 2), and the two isomers of the monofunctional monoadduct (spots 3 and 4; Fig. 5B). Unidentified spots x and y were also present in chick embryos treated with solvent alone (Fig. 5A), indicating that these most likely represent endogenous adducts (9, 10).

Table 1 compares the adduct levels of various DNA samples that were treated in vitro and in vivo with MMC. The most notable difference between in vitro chemical activation of MMC with SDT as compared to biochemical activation in vivo is the proportion of total bifunctional adducts to monofunctional adducts, indicating that monofunctional activation is a major contributor to MMC adduction in vivo. The amount of total MMC binding was approximately 100-fold lower in vivo than in vitro under these conditions. It is also interesting to note that the α isomer of the monoadduct was the predominant MMC adduct in rat hepatoma H4IIE cells in culture, whereas in chick embryo hepatocytes in vivo, this adduct represented only one-fifth of the total MMC adducts (Table 1).

The time course for the formation and removal of specific MMC-DNA adducts was examined over a 48-h period following a single dose of 30 μmol/kg MMC (Fig. 6). There was a close correlation between the time course for total MMC adducts as determined by postlabeling (Fig. 6) and the time course reported previously for radiolabeled porfiromycin binding in 14 DI chick embryo liver (8). It should be noted that the largest variability in postlabeling results occurred at the time points with the highest adduct levels and the highest number of replicate samples; therefore, this represents primarily in vivo interembryo variability rather than assay variability. As early as 15 min after MMC administration, all four MMC adducts were detected. Adduct levels dramatically increased within the first 2 h after treatment and then more gradually increased to maximal levels by 6 h. Interestingly, the bifunctional monoadduct and the β isomer of the monofunctional monoadduct were still at approximately one-half maximal levels by 24 h. Elevated levels of the β isomer of the monofunctional monoadduct remained through 48 h. The interstrand cross-link represented approximately 13–14% of the total MMC adducts formed in vivo up to 12 h after MMC treatment (Fig. 6) and also appeared to be preferentially lost from liver DNA between 12 and 48 h (Fig. 6C).

To mimic the repeated dosing that is more typical of human cancer chemotherapy, chick embryos were administered a second dose of MMC 24 h after the first dose and then assayed 24 h after the second dose. Appropriate solvent controls were included at each time of treatment because the embryo is also rapidly developing over this 48-h time period (see scheme in Fig. 7). The 15 DI embryos treated with MMC for 24 h had total adduct levels that were 1.7-fold greater

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$^5$R. M. Caron and J. W. Hamilton. Developmentally specific effects of the DNA crosslinking agent mitomycin C on phosphoenol pyruvate carboxy kinase gene expression in vivo: correlation with changes in chromatin structure within the promoter region of the gene, manuscript in preparation.
than 14 DI embryos treated for 24 h at body weight-equivalent doses, suggesting a greater activation of MMC at the older age (Fig. 7; P < 0.005). Treatment with MMC for 24 h at 14 DI, followed by a second 24 h treatment with MMC at 15 DI, produced a 2.1-fold greater adduct level than a single 24-h treatment at 14 DI (P < 0.005; Fig. 7). The levels of adducts in the two dose treatment were exactly additive of what was observed for 14 DI embryos treated with a single dose for 48 h and 15 DI embryos treated with a single dose for 24 h, suggesting that the first dose did not affect the adduct formation of the second dose. The relative proportions of specific adducts in all of these treatments were approximately the same (Fig. 7). MMC adduct levels were also compared in the liver, lung, and kidney of 14 DI embryos following a single 8-h MMC treatment. Total and individual MMC adduct levels were comparable in lung and kidney but were 60 and 58% those of the liver, respectively, although the relative proportions of specific MMC adducts were comparable in all three tissues (Fig. 8). These results are consistent with the higher metabolic capacity and blood flow of the liver as compared to lung and kidney but suggest that there are not large quantitative or qualitative differences in MMC metabolism or DNA adduct formation among these three major organs in vivo.

The effects of various biochemical modulators of specific Phase I and II activation and detoxification pathways were examined to determine the possible role of these pathways on MMC adduct formation in vivo (Fig. 9). Glutathione, which is a phenobarbital analogue, was used to principally induce the major phenobarbital-inducible chicken cytochrome P450 genes, i.e., CYP2H1 and CYP2H2 (15, 22). TCBinduced to principally induce the major dioxin-inducible chicken P450 genes, i.e., CYP1A4 and CYP1A5 (14, 23). Dexamethasone was used to principally induce several glucocorticoid-inducible chicken P450 CYP3A isoforms (24). BSD and EA were used to principally induce several glucocorticoid-inducible chicken P450 genes, i.e., CYPÃŒA4 and CYP1A5 (14, 23). Dexamethasone was also shown to increase in all four adducts, resulting in a 1.7-fold increase in total MMC adducts (P < 0.05; Fig. 9). Dexamethasone was also shown to increase GSH levels by approximately 2-fold (Fig. 10). However, each of these agents was shown previously to be effective at modulating these pathways in the chick embryo liver, although they each cause other pleiotropic effects on Phase I and II metabolism (reviewed in Ref. 25), including changes in DTD and GSH levels, which were also measured following each of these pretreatments (Fig. 10). MMC was administered at a lower dose of 15 µmol/kg in these experiments to avoid possible combined toxicity with some of these agents. None of these pretreatments had any significant effect on total or individual MMC adduct levels (Fig. 9) with the exception of dexamethasone, which caused a proportional increase in all four adducts, resulting in a 1.7-fold increase in total MMC adducts (P < 0.05; Fig. 9). Dexamethasone was also shown to increase GSH levels by approximately 2-fold (Fig. 10). However, BSO and EA also suppressed liver levels to a comparable extent as dexamethasone, and glutathione increased GSH levels to a comparable extent as NAC (approximately 1.3-fold), whereas these treatments had no significant effect on MMC adduct levels, suggesting that the dexamethasone effect was not principally due to changes in GSH per se.

DISCUSSION

Previous methods used to detect MMC-DNA adducts have included UV-vis absorbance combined with HPLC (1, 26), binding of radiolabeled MMC or porfiromycin (8, 27, 28), and DNA alkaline elution (29). However, each of these methods is limited by either lack of sensitivity or specificity or limitation to only one or a few types of MMC-DNA adducts. Previous attempts to obtain antibodies against specific MMC-DNA adducts have been unsuccessful, most likely due to the nondistorting nature of the MMC interstrand cross-link and monoaducts in helical DNA that makes these complexes relatively nonantigenic (12). Thus, no good method existed for the detection of the low levels of specific MMC-DNA adducts that occur in cell culture or in vivo experiments at physiologically relevant doses. Previous attempts to use 32P-postlabeling to detect MMC-DNA adducts have met with limited success, primarily because the specific MMC-DNA adducts had not yet been well characterized at the time of these studies (30–32). As a result, there were problems with the assay, such as incomplete enzymatic digestion, poor resolution by TLC, and inability to characterize and definitively identify the adduct spots. Recently, a new 32P-postlabeling procedure was described that was initially optimized to detect the major MMC monofunctional (α isomer) monoaduct (11). This technique has been extended to examine other MMC-DNA adducts in vitro and in vivo, including the β isomer of the monofunctional monoaduct, the bifunctional monoaduct, and the interstrand cross-link. Unfortunately, the intrastand cross-link does not appear to be a substrate for the first two enzymatic reactions of this postlabeling procedure; thus, a different methodology may be required to examine this other MMC cross-link.

The four major MMC-DNA adducts detectable by this assay were observed both at the 15 and 30 µmol/kg MMC doses in chick embryo liver, lung, and kidney in vivo, demonstrating the sensitivity and specificity of this method (maximum total adducts/nucleotide, ~14 µmol/mol at 30 µmol/kg, 6 h). These adducts were identified as the interstrand cross-link (spot 1), the bifunctional monoaduct (spot 2), and two isomers of the monofunctional monoaduct (α and β, spots 3 and 4, respectively), based on use of authentic standards and/or modifications in the reducing reaction that favor formation of specific adducts as described previously (1, 11, 13, 19–21). A new spot was also observed (spot 5), which was only present in in vitro samples under chemical reduction and which appeared to increase in intensity with increasing storage of samples prior to analysis. The nature of this spot is not known but is assumed to represent either an artifact of the SDT reduction conditions and/or a degradation product of the interstrand cross-link. Interestingly, a higher mutation frequency has been

Table 1 Summary of maximal MMC-DNA adduct levels in various DNAs reacted with MMC in vitro and in vivo as detected by 32P-postlabeling

<table>
<thead>
<tr>
<th>% GC content</th>
<th>87-bp DNA fragment</th>
<th>Chick embryo liver</th>
<th>H4IIE cell line</th>
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<td>41</td>
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<td>1580</td>
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<td>25.1</td>
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<td>% monofunctional monoaduct γ</td>
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<td>15.9</td>
<td>24.2</td>
</tr>
</tbody>
</table>

*Biocultural conditions using SDT under argon.
*Fourteen DI chick embryo liver, treated with 30 µmol/kg MMC in vivo: maximum adduct levels shown above were observed at 6 h (n = 5).
*Rat hepatoma cell line H4IIE, treated with 0.1 µmol MMC in 95% air, 5% CO2: maximum adduct levels shown above were observed at 2 h (n = 2).
*ND, not detected.

6 J-M. Yuann and J. W. Hamilton. Effects of modulating ascorbate and glutathione levels on chromium-DNA binding and formation of chromium (V) in liver and kidney of chromium (VI)-treated OOS rats in vivo, manuscript in preparation.

observed in SDT-treated plasmid DNA in the absence of MMC as compared to untreated DNA, indicating that these reaction conditions may be inducing other forms of DNA damage (11).

All four adducts were detected in chick embryo liver in vivo as early as 15 min after treatment with MMC, and maximal levels of adducts were observed at 6 h. It is worth noting that all four adducts were present at roughly comparable levels in liver and other tissues in vivo, whereas only the \( \alpha \) isomer monoadduct was detected in MMC-treated rat hepatoma H4IIIE cells in culture. This may be due to the large differences in total MMC adduct levels in these two systems under these conditions. Alternatively, these results may indicate that activation and MMC adduct formation is qualitatively different in culture and in whole animals, even within the same cell type, which may make it difficult to draw conclusions regarding MMC metabo-

The time course for formation and removal of the MMC interstrand cross-link correlated closely with time course for effects observed previously of MMC on inducible gene expression in chick embryo liver in vivo (8), suggesting that this adduct may be principally responsible for these effects. Similarly, the chromium(VI)-induced interstrand cross-link had been shown to be most closely correlated with effects of chromium(VI) on inducible gene expression in this system (6, 7). The cross-link was present at about 13–14% of total adducts during the first 16 h after MMC exposure, which is in approximately 5-fold excess of its predicted level based on the total

![Fig. 6. Time course for formation and removal of MMC-DNA adducts in chick embryo liver in vivo.](image)

A. Individual MMC adducts: •, spot 1 (interstrand cross-link); •, spot 2 (bifunctional monoadduct); A, spot 3 (monofunctional monoadduct, \( \alpha \) isomer); and 4, spot 4 (monofunctional monoadduct, \( \beta \) isomer). B, total MMC adducts. C, interstrand cross-link as a percentage of total MMC adducts.

![Fig. 7. Effect of various treatment regimens...](image)

A. 14 DI, 15 DI, and 16 DI treatment schemes. B, total MMC adducts; C, individual MMC adducts: columns from light to dark (left to right) represent spot 1 (interstrand cross-link), spot 2 (bifunctional monoadduct), spot 3 (monofunctional monoadduct, \( \alpha \) isomer), and spot 4 (monofunctional monoadduct, \( \beta \) isomer), respectively.
therefore, possible that the effects of MMC on inducible genes may in role in gene regulation (34). It has been shown that methylated CpG sites are preferentially cross-linked by MMC in vitro (35). It is, large part be due to the high cross-linking efficiency of MMC at CpG sites that are clustered within the promoters of these inducible genes, thereby preferentially “targeting” MMC effects to this class of genes. Such a mechanism could account for the strong and selective effects of MMC on gene expression, even at very low doses in vivo.

The MMC interstrand cross-link also appears to be preferentially lost from the genome in vivo, because the proportion of cross-link to total adducts decreased significantly between 16 and 48 h after MMC treatment. Although the chick embryo liver is rapidly dividing during this period, this loss cannot be explained by simple dilution resulting data replication and tissue growth, because detailed analysis of the proportion of the total cross-links has been reported recently to severely compromise phage survival in a bacterial mutagenesis assay (40). However, recent studies in cultured mammalian cells have demonstrated that the MMC cross-link is repaired within 24 h after drug treatment (41), and the results presented here indicate that this lesion is also preferentially repaired in chick embryo liver in vivo, with approximately 75% of the total cross-links being removed within 18 h of attaining maximal levels. Similarly, chromium(VI)-induced DNA-protein and DNA interstrand cross-links have been shown to be rapidly and specifically removed from chick embryo liver DNA in vivo (42). Thus, preferential repair of cross-links may represent a survival strategy for normal dividing tissues, and this may be particularly true in developing systems such as the chick embryo. In contrast, it is interesting to note that there were significantly elevated levels of spot 4 (tentatively identified as the β isomer of the monofunctional monoadduct) relative to the other adducts by 48 h after MMC treatment. These results suggest that this adduct may not be easily detected by DNA damage recognition and repair enzymes; therefore, this adduct may represent a promutagenic lesion.

The biotransformation of MMC in vivo appears to be very complex. A variety of enzymes have been implicated in the reduction of mitomycin antibiotics to reactive species, including NADPH cytochrome c reductase (43-45), DTD (45, 46), xanthine oxidase (43, 44), xanthine dehydrogenase (47-49), cytochrome P-450 (50), and NADH cytochrome B5 reductase (51). It had been postulated previously that DTD is one of the principal pathways of MMC activation in vivo, based primarily on in vitro and cell culture experiments using the genomic CpG sites available and assuming a statistical distribution of adducts. CpG dinucleotides are also the sites of 5-methylcytosine methylation and are significantly underrepresented in the eukaryotic genome as a whole but are also clustered at much higher-than-predicted levels within certain promoters, where they likely play a key role in gene regulation (34). It has been shown that methylated CpG sites are preferentially cross-linked by MMC in vitro (35). It is, therefore, possible that the effects of MMC on inducible genes may in large part be due to the high cross-linking efficiency of MMC at CpG sites that are clustered within the promoters of these inducible genes, thereby preferentially “targeting” MMC effects to this class of genes. Such a mechanism could account for the strong and selective effects of MMC on gene expression, even at very low doses in vivo.

The MMC interstrand cross-link also appears to be preferentially lost from the genome in vivo, because the proportion of cross-link to total adducts decreased significantly between 16 and 48 h after MMC treatment. Although the chick embryo liver is rapidly dividing during this period, this loss cannot be explained by simple dilution resulting from DNA replication and tissue growth, because this could only account for about 25% of the loss of this adduct (36, 37) and because the other adducts are not lost at the same rate over this time period. It has generally been assumed that interstrand cross-links are more difficult for the cell to repair than are monoadducts (38, 39). Cross-links are considered lethal to cells if left unrepaired, and the preferential killing of rapidly dividing cells by cross-linking agents is believed to be primarily a result of formation of this lesion (4, 5). In support of this, formation of the MMC cross-link has been reported recently to severely compromise phage survival in a bacterial mutagenesis assay (40). However, recent studies in cultured mammalian cells have demonstrated that the MMC cross-link is repaired within 24 h after drug treatment (41), and the results presented here indicate that this lesion is also preferentially repaired in chick embryo liver in vivo, with approximately 75% of the total cross-links being removed within 18 h of attaining maximal levels. Similarly, chromium(VI)-induced DNA-protein and DNA interstrand cross-links have been shown to be rapidly and specifically removed from chick embryo liver DNA in vivo (42). Thus, preferential repair of cross-links may represent a survival strategy for normal dividing tissues, and this may be particularly true in developing systems such as the chick embryo. In contrast, it is interesting to note that there were significantly elevated levels of spot 4 (tentatively identified as the β isomer of the monofunctional monoadduct) relative to the other adducts by 48 h after MMC treatment. These results suggest that this adduct may not be easily detected by DNA damage recognition and repair enzymes; therefore, this adduct may represent a promutagenic lesion.

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ever, more recent studies have suggested that DTD does not play a role in MMC activation.

MMC adducts (57, 58). A number of cell culture studies have also indicated that DTD activity is not principally due to changes in GSH. Dexamethasone is a synthetic glucocorticoid hormone that induces specific CYP3A isoforms (24), as well as phosphoenolpyruvate carboxykinase (64), tyrosine aminotransferase, and a number of other liver-specific genes (25). It will be interesting to examine these other pathways in more detail as a means of understanding MMC metabolism in vivo. In summary, we have developed a 32P-postlabeling method for MMC adduct detection and have demonstrated that this assay can be a highly sensitive and useful tool for examining the biological effects of MMC in vivo. Such studies will aid in understanding the pharmacokinetics and pharmacodynamics of MMC as well as addressing mechanistic aspects related to its metabolism and formation and repair of MMC-DNA adducts in vivo. The doses of MMC used in this study are roughly comparable to those used in human chemotherapy, and preliminary results indicate that this assay can detect the same adducts in cancer patients treated with MMC. Tumor samples from patients treated with a single dose of either 5 or 10 mg/m² of MMC exhibited all four adducts in approximately equal proportions to those seen in the chick embryo tissues in this study. Studies to accurately determine specific adduct levels in detailed time course studies in mice and human patients are presently under way. The overall goal of these studies is to use this approach to develop more effective uses of cancer chemotherapy agents in the treatment of human malignancies.

Fig. 10. Effect of various biochemical modulators on DTD activity and GSH content in chick embryo liver in vivo. Chick embryos were treated as described in Fig 9. DTD activity (A) and GSH content (B) were assayed as described in “Materials and Methods” in separate lobes of the same liver samples used for 32P-postlabeling. Data represent the means (or range) of values from two to four individual embryos; bars, SD.

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