Detection of Mitomycin C-DNA Adducts in Human Breast Cancer Cells Grown in Culture, as Xenografted Tumors in Nude Mice, and in Biopsies of Human Breast Cancer Patient Tumors as Determined by $^{32}$P-Postlabeling

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ABSTRACT

Mitomycin C (MMC) is a DNA cross-linking agent that has been used in cancer chemotherapy for >20 years. However, little is known either qualitatively or quantitatively about the relationship between formation and repair of specific MMC-DNA adducts and specific biological outcomes. The goal of this study was to examine formation and removal of specific MMC-DNA adducts in breast cancer cells using a $^{32}$P-postlabeling assay in relation to cytotoxicity and other biological end points. MMC-DNA adducts were measured in cultured human metastatic MDA-MB-435 cells, in the same cells xenografted as a mammary tumor in nude mice, and in metastatic tumor biopsies obtained from human breast cancer patients undergoing MMC-based therapy. MMC adducts corresponding to the CpG interstrand cross-link, the MMC-G bifunctional monoadduct, and two isomers of the MMC-G monofunctional monoadduct were detected in most samples. Despite similarities in the overall patterns of adduct formation, there were substantial differences between the cultured cells and the in vivo tumors in their adduct distribution profile, kinetics of adduct formation and removal, and relationship of specific adduct levels to cytotoxicity, suggesting that the in vivo microenvironment may be clinically relevant because patients differ widely in their tolerance and sensitivity to MMC and other chemotherapy agents.

INTRODUCTION

The anticancer drug MMC$^3$ is a bifunctional cross-linking agent that requires chemical or enzymatic reduction to form covalent adducts with DNA principally at the N$^2$ position of guanine (G), forming monofunctionally and bifunctionally alkylated G-MMC monoadducts, and G-MMC-G interstrand and intrastrand cross-links at CpG and GpG sites, respectively (reviewed in Refs. 1 and 2). 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. It is generally believed that the interstrand cross-link is principally responsible for the cytotoxic and anti-tumor activity of MMC (3, 4), but formal evidence for this is still lacking. The relationship of particular MMC-DNA adducts to the specific biological outcomes such as alterations in DNA replication, RNA transcription, cell killing, and other toxic side effects of MMC is still poorly understood. This relationship may be clinically relevant because patients differ widely in their tolerance and sensitivity to MMC and other chemotherapy agents.

The balance between toxicity to normal tissues and killing of tumor cells is likely to be strongly influenced by differences in the types of DNA damage, their relative and absolute amounts, and the rates of formation and repair of these lesions between normal and cancerous cells. We and others have hypothesized that specific MMC-DNA adducts are responsible for individual biological effects, such as cell killing, toxicity, and tumor cell resistance or sensitivity. We previously described the optimization of a $^{32}$P-postlabeling assay using MMC-DNA standards, and we validated this assay by examining MMC-DNA adduct formation and removal in the chick embryo in relation to several biological end points (4). This previous study demonstrated that $^{32}$P-postlabeling is capable of detecting all of the MMC-DNA adducts, with the exception of the intrastrand cross-link (which is a poor substrate for T4 kinase), with high sensitivity. The goal of the current study was to compare the amount, type, distribution, and rate of formation and removal of specific MMC-DNA adducts in human metastatic breast cancer cells either grown in culture or xenografted into the mammary fat pad of nude mice in relation to cytotoxicity and other end

Received 7/21/00; revised 12/21/00; accepted 1/6/01.

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1 Supported by grants from the National Cancer Institute, NIH (CA9902, to J. W. H.), National Institute of Environmental Health Sciences, NIH (ES07373, to J. W. H.), the Norris Cotton Cancer Center (to A. J. W.), the Hitchcock Foundation (Award 250-401, to A. J. W.), and Bristol-Myers Squibb (to J. W. H.). Support for the Dartmouth College Molecular Biology Core Facility was provided by the Norris Cotton Cancer Center Core Grant (National Cancer Institute, NIH, CA23108) and the National Institute of Environmental Health Sciences, NIH (ES07373).

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3 The abbreviations used are: MMC, mitomycin C; HPLC, high-performance liquid chromatography; Pgp, P-glycoprotein.
points. In addition, we examined MMC-DNA adducts in biopsies of metastatic tumors from human breast cancer patients undergoing MMC-based therapy.

MATERIALS AND METHODS

Preparation of MMC-DNA Adduct Standards. MMC-DNA standards for the major monofunctional monoadduct and the interstrand cross-link were prepared as described (4, 5) and used to identify adducts (i.e., autoradiographic “spots”) from biological samples. In addition, bifunctionally monoadducted dGpC was obtained as a gift from Dr. Maria Tomasz (Hunter College, New York, NY) and was labeled with [γ-32P]ATP before digestion with snake venom phosphodiesterase, as described below. MMC-modified salmon sperm DNA produced under bifunctional activation conditions (4) was also used as a positive control for further adduct confirmation. The appropriate negative control from each biological model system, i.e., DNA isolated from saline-treated mice, cells treated with media alone, or patient biopsies taken just before MMC chemotherapy, were always assayed at the same time as MMC treatment groups.

MDA-MB-435 Cell Treatment. The MDA-MB-435 human breast cancer cell line was cultured as previously described (6). Cells were treated at 30–40% confluence with 0.1 mM MMC [−LD10 (6)] in serum-free medium. To avoid extended serum deprivation and to better simulate first-pass pharmacokinetics of drug exposure, the medium was changed at 4 h, at which time cells were either harvested or allowed to incubate further. In vivo, MMC is rapidly absorbed by the cells, i.e., within 1 h, and thus 4 h are more than sufficient for MMC to be absorbed and metabolized by the cells (Ref. 7 and references within). At various times after MMC treatment (0, 4, 12, 24 h) flasks were washed with cold PBS, and DNA was isolated as described below. A colony formation assay was used to determine cell survival after treatment with 0–5.0 μM MMC (6).

Treatment of Mice. Female Ncr-nude athymic mice (National Cancer Institute breeding stock; Charles River Corp., Wilmington, MA) were injected with MDA-MB-435 human breast cancer cells (2 × 10^6 cells) directly in the right lateral mammary fat pad, and tumors were allowed to grow for ~2 weeks, as previously described (8–10). MMC (1–4 mg/kg) was administered i.p. in 100 μl of sterile saline as previously described, and mice were sacrificed after 6 h. In addition, mice were treated with MMC at 4 mg/kg and were euthanized at various intervals between 1 and 144 h. At each time point, liver and tumor were removed from both treated and untreated mice. In addition, samples from lung (a more oxygenated tissue), bone marrow (the site of dose-limiting toxicity, i.e., myelosuppression), and blood (a potential surrogate tissue), were obtained at selected time points (0, 6, 24 h). Control mice were treated with 100 μl of saline alone. Tissues were immediately frozen on dry ice followed by storage at −75°C.

Patient Enrollment, Treatment, and Biopsy Protocol. Patients with stage IV breast cancer and dermal metastases who had failed a standard therapy or therapies (and met other necessary criteria) were entered into an Institutional Review Board-approved pilot study and Phase I clinical trial at the Dartmouth-Hitchcock Medical Center as described (Ref. 11; Table 1). Eligibility criteria also required a minimum of 4 weeks since the completion of prior therapies and recovery from any significant toxicities associated with those treatments. None of the patients was a current smoker. The dermal metastases from these patients were highly accessible to punch biopsies and therefore were a convenient model in which to study adducts before and during their therapy. All patients were given a single dose of MMC at 5 or 10 mg/m². A 4-mm punch biopsy of a dermal metastasis was obtained just before MMC treatment, and two additional biopsies were obtained after MMC at various times up to 144 h after treatment. For each patient, the time point preceding treatment with MMC proved critical in assessing their preceding adduct patterns, which are most likely due to other exposures. Six breast cancer patients with adenocarcinomas from this Phase I study were evaluated for MMC-DNA adducts. All samples were snap frozen and stored at −75°C until the time of DNA isolation.

Isolation of Genomic DNA. Mouse and human tissues were frozen and homogenized via mortar and pestle in liquid nitrogen and then transferred to Qiagen’s G2 lysis buffer (800 mM guanidine-hydrochloride, 30 mM EDTA, 30 mM Tris hydrochloride, 5% Tween 20, and 0.5% Triton X-100, pH 8.0; Qiagen, Chatsworth, CA) containing RNase A (0.2 mg/ml). Blood and bone marrow were also lysed in the same G2 buffer. To maintain continuity between different biological systems, the MDA-MB-435 cells were harvested from their flasks and lysed in this same G2 buffer. All samples were passed through an 18-gauge needle to break up clumps, 20 mg/ml protease was added, and genomic DNA was incubated and then isolated as described by the manufacturer (Qiagen). DNA was resuspended in water, quantified by spectrophotometry, and stored at −75°C. One-fourth of a mouse liver or tumor sample typically yielded 200–500 μg of DNA, and the human tissue typically yielded 10–50 μg of DNA.

32P-Postlabeling Assay for MMC-DNA Adducts. 32P-Postlabeling was performed as described (4). Briefly, an aliquot of DNA equal to 2.0–3.5 μg of each sample or 1 ng of standards was digested with nuclease P1 and prostatic acid phosphatase (Sigma, St. Louis, MO). The digestion was terminated by neutralizing the pH with Tris, an aliquot was reserved for HPLC, and the remainder was 32P-labeled with T4 kinase (50 μCi [γ32P]ATP per sample). Unincorporated [γ32P]ATP that was converted to 32P3P by apyrase was removed from the labeled adducts using a C18 solid phase extraction column (SepPak Vac; Waters, Milford, MA). The 32P-labeled adducts were eluted from the columns with 50 mM TEAA/CH3CN (50/50 v/v), dried on a Speed Vac (Savant, Farmingdale, NY), and further digested with snake venom phosphodiesterase I (Sigma). Adducts were separated by two-dimensional TLC on polyethyleneimine cellulosic plates (Machery-Nagel, Alltech, Deerfield, IL) using 1 M NaH2PO4, pH 6.5 (D1) and 1.3 M lithium formate/2.1 M urea, pH 3.5 (D2) and were visualized by autoradiography, cut from TLC plates, and quantified by liquid scintillation. The amount of adducts was calculated by dividing the radioactivity in each spot by the specific activity of the [γ32P]ATP (determined exactly with each lot) used in the kinase reaction. The amount of adducts was then divided by the total nucleotide content, which was determined most accurately by HPLC (Hewlett Packard 1090; Beckman Ultrasphere ODS reversed phase column, 5 μm, % mm × 25 cm; flow rate, 0.9 ml/min) using a standard

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nucleoside mix to calculate the total nucleoside content of each sample (4). HPLC analysis was also used for confirmation of complete digestion after the nuclease P1-prostatic acid phosphatase step, and for confirmation of lack of RNA contamination.

RESULTS

Specific MMC-DNA adducts were identified by 32P-post-labeling in three different but related biological systems: in MDA-MB-435 human breast cancer cells grown as a monolayer in culture; in these same cells grown as a xenografted tumor in the mammary fat pad of nude mice; and in tumor biopsies from human breast cancer patients. Although the same adducts were detected in each system, each system also showed differences in their adduct distribution profile, kinetics of DNA adduct formation and loss, and relationship of specific adducts to cytotoxicity. Surprisingly, the greatest difference was between the MDA-MB-435 cells grown in culture versus as an in vivo tumor, suggesting that the in vivo microenvironment of these cancer cells may alter this relationship.

Mouse liver demonstrated a unique, endogenous adduct that was present both in control and treated animals (Fig. 1A). However, its location did not interfere with the detection of the MMC-DNA adducts (Fig. 1B). We observed all previously characterized major MMC adducts except the intrastrand cross-link, which does not appear to be a good substrate for T4 kinase (4). Previously, the identities of the interstrand cross-link (spot 1) and the α isomer of the monofunctional monoadduct (spot 3) were confirmed by cochromatography with standards (4). The identity of spot 2 has now been further confirmed by cochromatography with a bifunctionally monoadducted dGpC standard that was labeled with kinase followed by snake venom phosphodiesterase I digestion (data not shown). Consistent with these assignments, monofunctional activation conditions (i.e., xanthine oxidase/NAPDH) produced predominantly spots 3 and 4, and bifunctional reducing conditions (i.e., sodium dithionite) favored predominantly spots 1 and 2 (4). Based on these studies, spot 4 is most likely the β isomer of the monofunctional monoadduct. These same adducts were also clearly detected in the mouse tumor (Fig. 1, C and D). Frequently water instead of DNA served as an additional negative control to explain occasional unknown spots, which appear to be contaminants in the [γ32P]ATP.

The time courses for formation and removal of MMC-DNA adducts were also measured in the different biological systems. In all cases, the adducts were formed rapidly upon treatment with MMC and slowly disappeared after treatment (4).

Fig. 1 32P-postlabeling of mouse liver and breast tumor xenograft after treatment of MMC in vivo. Xenografted nude mice were treated with saline alone (A and C) or 4 mg/kg MMC (B and D) as described in “Materials and Methods.” DNA was isolated from liver (A and B) and tumors (C and D) and 32P-postlabeled as described in “Materials and Methods.” Liver exhibited a characteristic endogenous spot present in both saline- and MMC-treated mice (A and B). Using authentic standards and other confirmatory data, four spots have been identified as the MMC interstrand cross-link (arrow 1), bifunctional monoadduct (arrow 2), and two isomers (α and β) of the monofunctional monoadduct (arrows 3 and 4, respectively). The MMC intrastrand cross-link is a poor substrate for T4 kinase and cannot be detected by this assay. The slightly shifted mobilities of spots in C and D were due to a new lot of TLC plates and new separation buffers (compare D with B); however, standards run with each experiment aided in spot identification.
DNA adducts were compared in mouse liver, the MDA-MB-435 breast tumor xenografts, and cultured MDA-MB-435 cells. In liver, adduct levels reached maximal levels by 1 h and remained relatively constant between 1 and 48 h (Fig. 2A), suggesting a slow rate of repair. In contrast, in the breast tumor xenografts, maximal adduct levels were not achieved until 6 h, and these levels decreased substantially between 6 and 12 h (Fig. 2B). This suggests that the activation of MMC is slower in the tumor xenograft, but the repair of these adducts occurs relatively rapidly. Surprisingly, the MDA-MB-435 cells grown as a monolayer in culture displayed slower adduct kinetics (i.e., slower bioactivation of MMC) than the same cells growing as a xenograft tumor in mice. Maximal adduct levels were reached at 12 h after treatment but also decreased substantially between 12 and 24 h (Fig. 2C). Thus, human breast cancer cells grown in a monolayer and as a tumor xenograft demonstrated similar, rapid rates of repair of MMC-DNA adducts. Due to the rapid doubling of these cells in culture, it was not possible to conduct longer term studies of adduct levels without splitting the cells, which is expected to alter adduct levels and their repair/loss.

The distribution of adducts was evaluated at three different doses of MMC in mouse liver and tumor xenografts (Fig. 3). Adduct levels were essentially the same at 1 and 2 mg/kg but increased substantially at 4 mg/kg MMC. Interestingly, the relative individual adduct levels also changed between 2 and 4 mg/kg in the liver (Fig. 3). Monofunctional monoadducts predominated in the tumor tissue. This does not support the hypothesis that the tumor is more hypoxic than normal tissue because reducing conditions favor bifunctional adducts (i.e., bifunctional monoadducts and cross-links). The dose of 4 mg/kg produced no observable toxicity to the animals in this or a previous study (8), and, despite the higher adduct burden in the tumor observed here, we did not previously observe an alteration in tumor growth over time (8).

MMC adduct formation and loss were compared in mouse liver, lung, blood, bone marrow, and tumor xenografts at the time of maximal adduct levels (1 or 6 h) and at 24 h (Fig. 4). Total adduct levels in the lung and bone marrow were lower than in all of the other tissues. Adduct levels were lowest in liver.
Comparison of MMC-DNA adducts at early and late time points in the mouse xenograft in vivo. Xenografted mice were treated with 4 mg/kg MMC and tumors, livers, lung, bone marrow, and blood were removed at 1, 6, or 24 h. DNA was 32P-postlabeled as described in “Materials and Methods.” Each data point represents the mean ± SD of values from two to three individual mice. Blood samples from two mice were pooled preceding DNA isolation and postlabeling; data for this tissue represent the average of one pooled sample repeated twice. All four MMC adducts were detected in each tissue except bone marrow and blood. MMC adducts: columns (light to dark, left to right) represent spot 1 (interstrand cross-link), spot 2 (bifunctional monoadduct), spot 3 (monofunctional monoadduct, α isomer), and spot 4 (monofunctional monoadduct, β isomer), respectively.

Bone marrow and did not appear to decrease significantly between 6 and 24 h. Only the bifunctional monoadduct and one of the two monofunctional monoadducts were detectable in bone marrow. DNA from circulating WBC contained substantially more MMC adducts than the other tissues. Although the interstrand cross-link was detectable at 6 h, none was detectable at 24 h. The two monofunctional monoadducts were observed at higher concentrations in the blood than in any other tissue, suggesting a poorer reducing environment for MMC activation. These adduct concentrations did not decrease between 6 and 24 h. Thus, although MMC-DNA adducts are readily detectable in blood, this does not appear to be a good surrogate for assessing MMC adducts in tumor tissue.

Tumor DNA was analyzed for MMC adducts in biopsies of six breast cancer patients with dermal metastases that were readily accessible to punch biopsy. Background spots were observed in the human tumor samples before patient treatment with MMC (Fig. 5A, “control”), which presumably represent endogenous adducts and/or adducts from other chemotherapy agents in these heavily pretreated patients. In some cases, these interfered with the detection of one of the two MMC-DNA monoadducts. Also, not all MMC adducts were observed in all patients. Adduct levels were about 10-fold lower in humans receiving 5–10 mg/m² than in mice receiving 4 mg/kg MMC. The adduct levels and distributions in patients A–F all appeared similar (Fig. 6). No correlations were observed between the adduct profile and previous smoking history, prior chemotherapy, age, estrogen receptor status, or progesterone receptor status (Table 1). There was too much interindividually variability to allow pooling of data from different patients to construct a time course. However, a general trend was observed of moderately decreasing MMC adducts over the 96-h time course of these experiments. It is not known how long such adducts persist in patients, but based on these preliminary data it appears that MMC-DNA adducts persist longer in patient tumors than in the mouse xenograft tumors or in the cultured tumor cells. The six patients from this study all had infiltrating ductal cancer of the breast. It is possible that different tumor types may have different adduct distributions. This should be evaluated in future studies.

A final goal was to investigate the relationship among MMC dose, adduct formation, and cytotoxicity in the breast cancer cells in culture. Cells were treated with 0.01–5.0 μM MMC in parallel experiments examining colony-forming ability and MMC adduct levels. All adduct levels increased dramatically between 0.5 and 1.0 μM MMC but did not further increase between 1.0 and 5.0 μM (Fig. 7A). The relative levels of adducts differed between the same cells grown in culture or as tumors in mice (compare Figs. 4 and 7A). Bifunctional adducts predominated in culture (similar to mouse liver) whereas monofunctional adducts predominated in the xenografted tumors of these cells. MMC demonstrated a steep, almost threshold-like dose-response curve in cell culture in vitro and in vivo (Figs. 3 and 7A). By plotting the adduct data as a percent of total adducts formed at each dose (Fig. 7B), subtle changes in the profile of adducts can also be observed. For example, as the dose increases, a threshold effect occurs at which the percentage of interstrand cross-link decreases and the percentage of bifunctional monoadduct reaches a plateau.

Total MMC adduct levels correlated closely with cell survival in a colony formation assay (Fig. 8). In these MDA-MB-435 cells, a dose between 1 and 5 μM MMC was lethal, causing 20 × 10⁻⁶ total adducts/nucleotide (at 1 μM), of which 3 × 10⁻⁶ (15%) was represented by the interstrand cross-link. In comparison, maximum total MMC-DNA adduct levels obtained in breast cancer patient tumors (3.46 ± 0.76 × 10⁻⁷ total adducts/nucleotide, 0.76 ± 0.22% cross-links) at a dose of 10 mg/m² correspond to the equivalent of approximately 0.1 μM MMC in the MDA-MB-435 cells in culture. This would suggest that 10 mg/m² in humans may not cause sufficient DNA damage required for effective breast tumor cytotoxicity. However, significant clinical responses have been observed in some patients using MMC in breast cancer salvage regimens at doses of 10–20 mg/m². Because these samples were obtained from a Phase I clinical trial, prognosis information was not a goal of this study. In mice receiving 4 mg/kg MMC, higher adduct levels were observed in the tumor xenograft (2.59 ± 0.349 × 10⁻⁶ total adducts/nucleotide, 0.349 × 10⁻⁶ cross-links/nucleotide, or 13.5% cross-links) than mouse liver (1.29 ± 0.263 × 10⁻⁶ total adducts/nucleotide, 0.263 ± 0.04% cross-links). The adduct levels produced in the mice at this dose (4 mg/kg) produced adduct levels comparable with about 0.1–0.5 μM MMC in the MDA-MB-435

cells in culture. At this dose of MMC, no significant effect was observed on xenograft tumor growth in parallel studies with this mouse model (8). Thus, the relationship between MMC-DNA adducts and cytotoxicity in vivo appears more complex than the cell culture system.

**DISCUSSION**

DNA is postulated to be the principal target for a number of cancer chemotherapy drugs. However, despite intense investigation, we still have a poor understanding about the precise relationship between specific DNA adducts formed by DNA alkylating cancer chemotherapy agents and specific therapeutic and toxic end points in vivo. Characterizing the types of DNA adducts formed by these agents, and determining their biological effects will hopefully lead to both a more mechanistic understanding of drug action as well as the design of better chemo-therapeutic regimens. Precise measurement of DNA adducts from chemotherapeutic agents in vivo is also useful in determining the amount of drug that reaches a given target or nontarget cell, the extent and nature of drug activation versus detoxification, the persistence of adducts which reflects DNA repair capacity, and the apoptotic, antiapoptotic, and proliferative responses of cells. Several previous studies have attempted to attribute specific DNA adducts from other chemotherapeutic drugs with clinical activity or other biological effects by characterizing certain cell lines as models for prediction of patient adduct levels, tumor sensitivity, resistance to chemotherapy, and patient outcome (12–15). With respect to the mitomycins, [3 H]porfiromycin and [ 3 H]mitomycin DNA adducts (expressed as adducts/nucleotide) had previously been measured in EMT6 mouse mammary cells (16, 17), but due to limitations in the technology these adduct levels could not be directly compared quantitatively to the [3 H]porfiromycin adduct levels in mice bearing EMT6 tumors (expressed as cpm/mg tissue or grain counts) (7). The 32 P-postlabeling assay allows precise and sensitive quantitation of individual MMC-DNA adducts, allowing direct comparison of adduct levels in different biological systems.

We previously described the development and use of a 32 P-postlabeling assay to assess MMC-DNA adduct formation and removal in a model in vivo system, the chick embryo (4, 18). In the present study, we have used this assay to examine specific MMC-DNA adducts in relation to cytotoxicity in a human breast cancer cell line in culture, in these same cells transplanted as a tumor in nude mice, and in the tumor cells of patients receiving chemotherapeutic and subchemotherapeutic doses of MMC. MMC-specific adducts corresponding to the CpG interstrand cross-link, the MMC-G bifunctional monoadduct, and two isomers of the MMC-G monofunctional monoadduct were detected in human breast cancer patient tumors and xenografted mouse tumors, as well as in human breast cancer cells grown in culture. We did not detect additional adducts (i.e., adducts X and Y) that have recently been reported (19, 20). However, these additional minor adducts were characterized in cells treated with

Fig. 5 32 P-postlabeling of human breast tumor tissue after treatment with MMC in vivo. DNA was isolated from biopsies of dermal metastases of human breast cancer patients and 32P-postlabeled as described in “Materials and Methods.” For each patient, biopsies were taken just before MMC treatment and at one or two time points after treatment with 5 or 10 mg/m2 MMC. Each patient had a unique pattern of preexisting background spots (A). In most cases after treatment with MMC (B), three spots could be detected: interstrand cross-link (arrow 1), bifunctional monoadduct (arrow 2), and the β isomer of the monofunctional monoadduct (arrow 4). Spot 3 (the α isomer of the monofunctional monoadduct) could not be detected due to interference of an endogenous spot present in all human samples.

Fig. 6 Comparison of MMC-DNA adducts in human breast tissue after treatment with MMC in vivo. Samples were postlabeled as described in Fig. 5. MMC adducts: columns (light to dark, left to right) represent spot 1 (interstrand cross-link), spot 2 (bifunctional monoadduct), and spot 4 (monofunctional monoadduct, β isomer), respectively.
The results from our study indicate the usefulness of this assay for assessing MMC-induced DNA damage and repair and its relationship to chemotherapeutic response in cell culture and in animal models and human patients in vivo. On the one hand, MMC was clearly activated in all three systems and produced a similar spectrum of DNA adducts. On the other hand, there were also qualitative and quantitative differences between the cell culture system and the mouse xenografted tumors, using the same cells, in terms of relative adduct levels, total adduct levels, percentage of interstrand cross-links, kinetics of formation and removal of adducts, and the relationship of these adducts to biological responses such as cytotoxicity and tumor growth. This suggests that the in vivo microenvironment of the cells may play a large role in their overall response to MMC, although the same types of adducts are formed. Similarly, it is interesting to note the substantially lower adduct levels in human patients’ tumors than in the mouse xenografts at roughly comparable doses (in terms of systemic toxicity).

The finding that bioreductive drugs such as MMC are strongly influenced by microenvironmental factors is not surprising. These results are consistent with other studies demonstrating that factors such as pH, degree of oxygenation, available NADPH or NADH, and the activity of oxidoreductases within and most likely surrounding tumors in vivo, as well as in vitro, all modulate MMC alkylation and cytotoxicity (21–27). Because similar rates of MMC-DNA adduct loss (i.e., repair) were observed in breast cancer cells grown in a monolayer and as a tumor xenograft, our data suggest that the rate of bioreduction of MMC is most strongly influenced by the microenvironment of the cells.

Keyes et al. (7) had previously used a much higher dose of 20 mg/kg [3H]porfiromycin (an N-methyl analogue of MMC with similar activity) in BALB/c mice bearing EMT6 mouse mammary tumors, principally due to limitations in the specific activity of the radiolabeled drug. In that study, similar pharmacokinetics was observed in all tissues studied, and adduct levels were approximately the same between 4 and 24 h after exposure. By using the [32P]-postlabeling assay, we were able to quantify individual MMC-DNA adducts and use a much lower dose of MMC (4 mg/kg) which is roughly equivalent to a conventional human chemotherapy dose. Using this approach, we observed

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<td></td>
<td>CMF for 3 cycles</td>
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<td></td>
<td>Megestrol acetate</td>
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<tr>
<td>E</td>
<td>69</td>
<td>ER+/PR+</td>
<td>CAF for 11 cycles</td>
<td>n/a achieved only partial response</td>
<td>1 pack/day for 40 yr</td>
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<td></td>
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<td>CMF for 4 cycles</td>
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<td>MMC for 1 cycle</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Bispecific antibody</td>
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<td></td>
<td></td>
<td></td>
<td>Tamoxifen</td>
<td></td>
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<tr>
<td>F</td>
<td>46</td>
<td>ER+/PR+</td>
<td>AC for 3 cycles</td>
<td>7 mo</td>
<td>No history of tobacco use</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High dose CTX, TPA, BCNU</td>
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<td>Tamoxifen</td>
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<td>Taxol for 9 cycles</td>
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<td>Anastrozole</td>
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</tbody>
</table>

Table 1 Breast cancer patient histories

- ER, estrogen receptor; PR, progesterone receptor; AC, Adriamycin, cyclophosphamide; CAF, cyclophosphamide; Adriamycin, 5-fluorouracil; CAFP, cyclophosphamide, Adriamycin, 5-fluorouracil, prednisone; CMF, cyclophosphamide, methotrexate, 5-fluorouracil; CTX, cyclophosphamide; BCNU, carmustine; MMC, mitomycin C; TPA, thiota; NCI, National Cancer Institute; unk, unknown.
tissue-specific differences in MMC-DNA adduct distribution and pharmacokinetics, suggesting differences in drug activation and repair. The relative levels of total tissue adducts were liver > tumor > blood. The rapid formation of adducts in all mouse tissues was consistent with the pharmacokinetic data (half-life, 15–40 min) in mice (7, 28) and in humans (29, 30). Adducts reached maximal levels more rapidly in liver than in tumor, perhaps due to the higher metabolic activity and/or higher blood flow of liver. Conversely, adducts were removed much more rapidly by tumor tissue, presumably through DNA repair mechanisms because there was little or no inhibition of tumor growth with this treatment (8). Based on the low relative adduct levels in bone marrow observed here, it is difficult to explain the major clinical side effect of MMC, i.e., myelosuppression, unless these adduct levels are not repaired and/or accumulate with consecutive dosages, or these stem cells are hypersensitive to this type of DNA damage. Similarly, Keyes et al. (7) observed low levels of porfiromycin adducts in bone marrow and speculated that the hematopoietic system may be more sensitive to these MMC lesions.

Interestingly, the same human breast cancer cells grown as a monolayer in culture formed maximal adduct levels much more slowly than the cells grown as a three-dimensional tumor in the mouse mammary fat pad, although the kinetics of adduct loss (i.e., repair) were similar. This suggests that the bioreduction of MMC is slower in culture due to factors such as oxygen tension, pH, endogenous oxidoreductase activity, and/or NADPH/NADH availability (22, 27). In the mouse, it is likely that other surrounding cells may assist in MMC activation by altering any of these factors and that the three-dimensional structure of the tumors themselves may influence the degree of oxygenation and drug uptake (e.g., via blood flow). However, although maximal adducts were achieved faster in the tumor xenograft, monofunctional monoadducts predominated, which are generally produced under more aerobic conditions. Thus, different factors appear to influence the rate of maximal adduct formation and the type of adducts formed. Such a rapid removal of MMC adducts in both systems was not observed in chick embryo liver (4), although it is a rapidly growing tissue that has an otherwise robust DNA repair capacity (4, 31, 32). In the chick embryo, the rate of adduct removal far exceeded the rate of cell doubling (4). Similarly, the loss of MMC adducts in the breast cancer cells in culture cannot be explained solely by dilution due to cell growth, because the doubling time of these cells is ~36 h.6 The growth of the mouse xenografted breast cancer cells was even slower, with a tumor volume doubling time of ~36 h.6

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time of ~14 days (8). It was surprising that the cells in culture had a larger proportion of bifunctional lesions compared with the mouse tumor, because these cells were not cultured under N₂ (i.e., hypoxic conditions), and tumors are generally believed to be in a hypoxic environment. Further studies are required to better characterize the relationship between specific adducts and cytotoxicity in the mouse xenograft model and in human chemotherapy patients, but clearly the in vivo situation was more complex in terms of adduct levels, the spectrum of adducts formed, and the kinetics of adduct formation and removal.

This study describes the first detection and identification of specific MMC-DNA adducts resulting from therapeutic doses of MMC in human cancer chemotherapy patients. Previous studies that used 32P-postlabeling to examine MMC-DNA adducts in rats (33) or in human patients (34) were inconclusive because of incomplete digestion of adducts to the mononucleoside level, an inability to identify specific adducts due to a lack of appropriate standards, and an incomplete knowledge of MMC chemistry at the time of those studies. The 32P-postlabeling assay described in this work was able to detect the major MMC-DNA adducts, with the exception of the intrastrand cross-link, in each biological system. Thus, MMC-DNA adducts could potentially serve as an intermediate marker of the biologically effective dose, i.e., a potential indicator for the host’s exposure and capacity for metabolic activation, detoxification, and DNA repair. We also detected several other major adducts in the mouse and human in vivo experiments by 13C-postlabeling, presumably representing both endogenous adducts (i.e., “I-compounds” (35)) and adducts resulting from exposure to other exogenous agents. Significant interindividual variation in MMC-DNA adduct levels was observed in the human breast cancer patients, as one might expect in a small number of heterogeneous individuals. This interindividual variation deserves further study in a much larger population. The patients in this study were part of a Phase I study; thus, the goal of that work did not include a measurement of patient response. Further descriptions regarding the toxicity of their treatments and degree of drug resistance will be reported elsewhere (3).

In this and in previous studies, we have observed a threshold-like dose-response both for MMC-DNA adduct formation and for MMC-induced biological effects. Adduct formation increased dramatically between 0.5 and 1.0 μM MMC in the breast cancer cells in culture and between 2 and 4 mg/kg in the xenografted tumors in vivo. Similarly, we previously observed little or no effect of 0.5–2.0 mg/kg MMC on Pgp expression in this in vivo xenograft tumor model, whereas there was a strong suppression of Pgp expression at 4 mg/kg MMC (8). We have also observed a similar threshold for Pgp suppression in tumors of human patients between 5 mg/m², at which there is little or no effect, and 10 mg/m², where we see a greater than 75% suppression in Pgp expression within 24 h of MMC treatment (11, 30, 36). The mechanistic basis for this apparent threshold is not clear but may have important implications for determining the effective dose in human therapies as well as understanding interindividual differences in therapeutic and toxic responses. Determining the mechanisms underlying the biological effects of MMC and other cancer chemotherapy drugs is fundamental for effective therapeutic treatment, with the overall goal of minimizing nontarget toxicities while optimizing the ability of these drugs to selectively kill cancer cells or otherwise modify their phenotype in a therapeutically beneficial way.

ACKNOWLEDGMENTS

We thank Dr. Rodney Page, Cornell University, Ithaca, NY, who was on sabbatical leave in our laboratory; Dr. Michael Ihnat, Duquesne University, Pittsburgh, PA; and Jean Lariviére for assistance in handling and treatment of the mice. We also thank Dr. Maria Tomasz, Hunter College, New York, NY, for her critical comments and willingness to provide the dGpC bifunctional monoadduct for further adduct confirmation. In addition, Dr. Alexander MacCubbin, Schering-Plough Research Institute, Kenilworth, NJ, was helpful in his technical advice and insightful interpretation of the data.

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Detection of Mitomycin C-DNA Adducts in Human Breast Cancer Cells Grown in Culture, as Xenografted Tumors in Nude Mice, and in Biopsies of Human Breast Cancer Patient Tumors as Determined by $^{32}$P-Postlabeling

Amy J. Warren, David J. Mustra and Joshua W. Hamilton

*Clin Cancer Res* 2001;7:1033-1042.

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