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Enzymatic and pH Modulation of Mitomycin C-Induced DNA Damage in Mitomycin C-Resistant HCT 116 Human Colon Cancer Cells

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SUMMARY

The effect of pH and oxygen on DNA alkylation by mitomycin C (MMC) was studied with cell fractions and intact cells. The cell lines used were the HCT 116 human colon cancer cell line and a MMC-resistant subline (HCT 116-R30A) that has 5% of the quinone reductase activity present in the parent cell line. Microsomal fractions of the two cell lines catalyzed MMC-DNA adduct formation only under anaerobic conditions with equal efficiency. However, the pH of the reaction controlled the production of four identified and two unidentified adducts. Soluble fractions from each cell source catalyzed MMC-DNA adduct formation under aerobic and anaerobic conditions similarly. At higher pH, limited DNA adducts were produced by MMC activated by soluble fractions from either cell source. At lower pH, more DNA adducts were obtained with MMC activated by the soluble fraction of HCT 116 cells than with that activated by the soluble fraction of HCT 116-R30A cells. Four of these adducts were identified as N^2-(2″β,7″-diaminomitosene-1″α-yl)-2′-deoxyguanylic acid, N^2-(2″β,7″-diaminomitosen-1″β-yl)-2′-deoxyguanylic acid, N^2-(10″-decarbamoyl-2″,7″-diaminomitosen-1″α-yl)-2′-deoxyguanylic acid, and N^2-(2″β,7″-diamino-10″-deoxyguanyl-N^2-yl-mitosen-1″α-yl)-2′-deoxyguanylic acid. Acidic intracellular pH enhanced the cytotoxicity of MMC for HCT 116 cells, decreasing the IC_{50} from 0.3 ± 0.04 μM to 0.1 ± 0.03 μM, but pH had limited effect on the cytotoxicity of MMC for HCT 116-R30A cells. When intracellular pH was decreased, interstrand DNA cross-linking by MMC increased to a greater extent in HCT 116 cells than in HCT 116-R30A cells. Only two DNA adducts, each at low intensity, were detected in HCT 116-R30A cells treated at pH 6.0 and 7.6 and in HCT 116 cells treated at pH 7.6. However, six radioactive spots were detected in HCT 116 cells treated at pH 6.0. Three of these adducts were identified. This is the first direct evidence that acidic intracellular pH enhances MMC-DNA adduct formation in tumor cells containing high quinone reductase activity. Results from this study further confirm that pH and not enzyme is the determining factor in the distribution of types of MMC-DNA adducts. This study also indicates that low intracellular pH enhances the activity of quinone reductase in reducing MMC, which is important for aerobic cytotoxicity of MMC against tumor cells with high concentration of quinone reductase.

The complex relationship between reductive activation of MMC and cytotoxicity has been a subject under investigation for some time. Both one-electron and two-electron enzymatic reductive pathways have been proposed as ways for MMC to be converted to active intermediates (1–5). From these investigations, two major factors, oxygen tension and pH, have been observed to play important roles in the outcome of enzymatic and chemical reduction of MMC.

One-electron activation of MMC to a semiquinone intermediate by microsomal NADPH-cytochrome P450 reductase (EC 1.6.2.4) (4) and xanthine oxidase (EC 1.2.3.2) (4) leads to two possible reaction pathways, depending on the oxygen tension. In the absence of oxygen, the semiquinone results in the formation of MMC metabolites and alkylation of cellular macromolecules. In the presence of oxygen, the semiquinone intermediate of MMC becomes involved in redox cycling, producing active oxygen species that are also cytotoxic (6–8). The cellular role of one-electron reductive systems under hypoxic conditions has been demonstrated by showing a correlation between preferential activation of MMC and porfiromycin and the enhanced cytotoxicity of these agents in hypoxic tumor cells (9, 10). On the other hand, two-electron reduction of MMC by DT-diaphorase (EC 1.6.99.1) is not affected by the presence or absence of oxygen (11). The presence of oxygen does not affect

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ABBREVIATIONS: MMC, mitomycin C; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(ethanesulfonic acid); BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; AM, acetoxymethyl ester; HBSS, Hanks' balanced salt solution; α (or β)N^2-G-MMC, N^2-2″β,7″-diaminomitosen-1″α (or β)-yl)-2′-deoxyguanylic acid; N^2-G-DMC; N^2-(10″-decarbamoyl-2″,7″-diaminomitosen-1″α-yl)-2′-deoxyguanylic acid; $(N^2\text{-G})_2$-MMC, N^2-(2″β,7″-diamino-10″-deoxyguanyl-N^2-yl-mitosen-1″α-yl)-2′-deoxyguanylic acid (adduct of two deoxyguanylic acids cross-linked at the N^2-position by mitomycin C); TLC, thin layer chromatography.

the ability of MMC-hydroquinone, the MMC intermediate of two-electron reduction, to form metabolites or to alkylate DNA (11–13).

An effect of pH on the metabolism of MMC has been observed during one-electron reduction by NADPH-cytochrome P450 reductase and xanthine oxidase (4) and electrochemical reduction (14). More recently, Siegel *et al.* (13) showed that two-electron reduction of MMC by DT-diaphorase was also pH dependent. In all cases, pH controlled the kinetics of enzymatic reduction of MMC and the profiles of metabolite formation. NADPH-cytochrome P450 reductase and xanthine oxidase reduced MMC more efficiently at higher pH, whereas their activities decreased 80% when the pH changed from 8.2 to below 6.5 (4). In contrast, DT-diaphorase reduced MMC more efficiently at lower pH than at higher pH (11, 13). The major metabolite produced at acidic pH by either one- or two-electron enzymatic reduction of MMC was 2,7-diaminomitosene (4, 11). In contrast, *trans*-2,7-diamino-1-hydroxylmitosene was the major product of one-electron enzymatic reduction at pH values above 7.8 (4). The interaction of DNA with MMC activated by one-electron and two-electron reductive enzymes was also pH dependent (12, 13, 15, 16). At the cellular level, an effect of pH on MMC cytotoxicity has been reported. Kennedy *et al.* (17) have shown MMC-induced DNA cross-linking and aerobic cytotoxicity to be enhanced by lowering intracellular pH in EMT6 cells.

The involvement of DT-diaphorase in the aerobic cytotoxicity of MMC was first demonstrated by inhibiting DT-diaphorase with dicumarol (18–20). More recently, correlation of DT-diaphorase activity with the development of MMC resistance under aerobic conditions in several cell sublines has been reported. These MMC-resistant sublines, including a MMC-resistant human skin fibroblast strain, 3437T (20, 21), and a MMC-resistant subline of Chinese hamster ovary cells (22), are all deficient in MMC activation activity and DT-diaphorase. Conversely, a subline with increased MMC sensitivity was developed from L5178Y cells and demonstrated a 24-fold increase in DT-diaphorase (23). The comparison of HT-29 and BE human carcinoma cells, which have high and low levels, respectively, of DT-diaphorase activity, also implicated DT-diaphorase in the reductive activation of MMC and MMC sensitivity (11).

We developed a MMC-resistant subline, HCT 116-R30A, from human colon carcinoma HCT 116 cells (24). This subline has 5% of the quinone reductase (DT-diaphorase) activity and equal levels of microsomal NADPH-cytochrome P450 reductase activity, compared with MMC-sensitive parent cells. In the current report, we present data that 1) identify DNA adducts produced by MMC that had been activated by cellular fractions of these two cell lines at different pH values and 2) correlate levels of quinone reductase activity in MMC-sensitive and -resistant cells with MMC-induced actions, including cytotoxicity, DNA interstrand cross-linking, and DNA adduct formation, which are enhanced at acidic pH values.

Materials and Methods

Reagents. MMC and porfiromycin were kindly supplied by the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). BCECF and BCECF/AM were from Molecular Probes, Inc. (Eugene, OR). Tetrapropylammonium hydroxide was from RSA (Ardsley, NY). Proteinase K (EC 3.4.21.14) was from Boehringer Mannheim (Indianapolis, IN). Nitrogen mustard (Mustargen; Merck Sharp & Dohme, West Point, PA) was used as its clinical formulation. All enzymes and reagents required for ^{32}P-postlabeling were the same as described by Yu and Pan (25). RNase A (EC 3.1.27.5), DNase I (EC 3.1.21.1), snake venom phosphodiesterase I (EC 3.1.4.1), and other biochemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell lines. HCT 116 human colon carcinoma cells were obtained from the American Type Culture Collection (CCL247) (Rockville, MD). A MMC-resistant subline, HCT 116-R30A, was isolated as described in our earlier publication (24). Maintenance and subculture of both cell lines were as described previously (24). All incubations, including maintenance and experiments of short duration, were carried out in incubators at 37° with 5% CO_2 and 95% humidity. HCT 116-R30A cells were about 5 times less sensitive to MMC than were HCT 116 parent cells when cells were exposed to MMC in McCoy's 5A medium containing 10% fetal calf serum (24). HCT 116-R30A cells contained 20 times less quinone reductase activity but an equal amount of microsomal NADPH-cytochrome P-450 reductase activity, compared with parent HCT 116 cells. A detailed comparison of the enzymatic activities in the two cell lines has been reported (24). Other cellular detoxification systems such as reduced glutathione, glutathione transferase, glutathione peroxidase, and catalase are all present at similar levels in the two cell lines.1

Adjustment and measurement of intracellular pH. A specific intracellular pH for HCT 116 and HCT 116-R30A cells were achieved by a method described by Kennedy *et al.* (17). HBSS media were prepared with HBSS plus 0.7 g/liter ammonium acetate, 10 mM MES, and 10 mM HEPES, the pH was adjusted to a range of 6.0 to 7.6 with 1.0 N NaOH, and the media were sterilized by filtration. Cells were equilibrated in these media for 60 min at 37° to obtain the corresponding intracellular pH.

Intracellular pH was measured following the method described by Wang *et al.* (26), by flow cytometric analysis of the pH-sensitive fluorescent dye BCECF, which was converted from BCECF/AM by intracellular esterases. Confluent cells were harvested and resuspended at 5×10^5 cells/ml in regular growth medium with 1.0 μM BCECF/AM. After incubation at 37° for 30 min to allow for uptake and hydrolysis of BCECF/AM, cells were recovered by centrifugation, washed, and suspended in the HBSS medium designated for use. Analysis of intracellular pH was obtained with a Becton Dickinson FACStar Plus flow cytometer (San Jose, CA) with argon ion lasers emitting at 200 mW at 488 nm and 100 mW at 568 nm. Fluorescence emission from intracellular BCECF was measured at 530 ± 10 nm (f_1) and 630 ± 22 nm (f_2). The ratio of f_1 and f_2 in each cell is proportional to pH. Before any experiment was conducted, a standard curve of intracellular pH with cells containing BCECF was obtained according to a method described by Kennedy *et al.* (17). The BCECF-loaded cells were washed and suspended in buffers containing 20 mM ammonium acetate and 0.2 M MES or HEPES, with pH adjusted to between 5.8 and 7.6. The cells, at 5×10^5/ml, were incubated for 60 min at 37° for pH equilibration and then analyzed for fluorescence to give the standard curve (Fig. 1).

Effect of intracellular pH on MMC action. To study the relationship of intracellular pH to MMC cytotoxicity, MMC-induced DNA cross-linking, and DNA adduct formation, cells were divided into two portions. Intracellular pH was measured in one cell portion after exposure to BCECF/AM in regular medium for 30 min. The other cell portion was not exposed to BCECF/AM. Both cell portions were then recovered, washed with HBSS medium at a designated pH, and resuspended at 5×10^5 cells/ml in the same HBSS medium. After pH equilibration for 60 min, MMC at different concentrations, or an equivalent solution for the control cells, was added and cells were exposed to drug for 60 min at 37°. Intracellular pH was measured at the beginning and at the end of MMC exposure with BCECF-loaded cells, and unloaded cells were used for analyses of the cytotoxicity, DNA cross-linking, and DNA adduct formation. Stability of the extra-

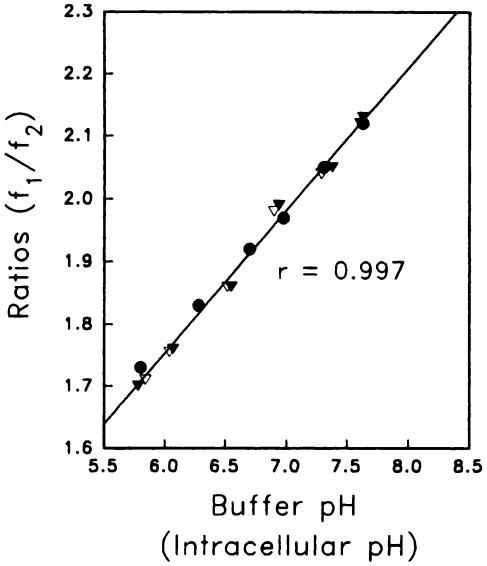

Fig. 1. Standard curve for the determination of intracellular pH of HCT 116 and HCT 116-R30A cells by fluorescence-activated cell sorter. Cells were prepared, as described in Materials and Methods, for pH analysis with a FACStar Plus flow cytometer. The fluorescence ratio (f_1/f_2) of the emission of BCECF at 530 ± 10 nm (f_1) and 630 ± 20 nm (f_2) was measured in aliquots of both cell lines that had been equilibrated in medium with a predetermined pH. The standard curve was obtained by plotting pH of the medium against fluorescence ratio. Data from three separate experiments are presented as a composite graph, with each experiment shown by a different symbol (●, ▼, ▽). Each *data point* represents the average of two separate measurements.

cellular pH of the HBSS medium was measured by pH meter after the addition of MMC and at the end of drug exposure, after removal of cells.

MMC cytotoxicity. Cytotoxicity of MMC for HCT 116 and HCT 116-R30A cells was assessed by a modified clonogenic assay (24). Cells were washed with HBSS medium at pH 6.0, 7.2, or 7.6 and were resuspended in the same medium. Preincubation for 60 min and drug exposure for 60 min at 37° were performed in these media in 25-cm^2 culture flasks. Control cells were incubated in the same medium without drug. After drug exposure, cells were recovered, washed with phosphate-buffered saline, and placed at regular medium for colony formation as described earlier (24). The IC_{50} for each study was calculated by median effect analysis with a program developed by Chou and Chou (27).

Alkaline elution measurements of interstrand DNA cross-linking. The alkaline elution method used for measuring interstrand DNA cross-linking was based on the procedures of Kohn *et al.* (28) and Hilton (29). Details of the method as implemented in our laboratory have been described previously (30).

Fractionation of microsomes and soluble cell extracts. Microsomes and soluble cell extracts of confluent HCT 116 and HCT 116-R30A cells were prepared according to the method of our earlier report (24), with some modification. Harvested cells (4×10^7 cells/ml) were lysed by three cycles of freezing and thawing in 50 mM MES at pH 6.0 or 50 mM Tris at pH 7.6. The cell homogenate was centrifuged at 10,000 × *g* for 20 min to remove large debris, mitochondria, and nuclei. The supernatant was further centrifuged at 144,000 × *g* for 60 min to separate microsomes and soluble enzymes. Microsomal fractions were washed twice with 50 mM MES at pH 6.0 or 50 mM Tris·HCl at pH 7.6 and were redissolved in buffer of the same pH in volumes equivalent to 4×10^7 cells/ml. The absence of xanthine oxidase and lack of possible contamination with mitochondrial enzymes in either fraction were checked as described (24).

Interaction of DNA and MMC activated by cellular fractions. Interaction of calf thymus DNA with MMC activated by microsomes or soluble extracts was conducted according to our previously published methods (15). Microsomes and soluble extracts of HCT 116 and HCT 116-R30A cells were used to activate MMC. The 1-ml reaction mixture contained 20 mM NaCl, 0.5 mM MMC, 1.0 mM NADPH (or NADH), 0.5 mg/ml calf thymus DNA in either 50 mM Tris at pH 7.6 or 50 mM MES at pH 6.0, and microsomes (or soluble extracts) at an amount equivalent to 2×10^7 cells. Activation by each enzyme at each pH value was conducted both aerobically and anaerobically at 37° for 60 min. Anaerobic conditions were achieved as described earlier (4, 15). Termination of the reaction and recovery of the alkylated DNA were carried out as described previously (15). Alkylated DNA was dissolved in 10 mM Tris·HCl, pH 7.4, for ^{32}P-postlabeling.

Drug treatment of cells and isolation of cellular DNA. Cells were harvested, washed once with cold HBSS medium (at pH 6.0 or 7.6), and resuspended to 2×10^6 cells/ml in HBSS medium of the same pH. Five milliliters of cell suspension were placed into 25-cm^2 flasks and preincubated at 37° for 60 min to equilibrate intracellular pH and extracellular pH. MMC, at a final concentration of 25 μM, or control medium was added, and incubation at 37° was resumed for an additional 60 min. After drug exposure, cells were recovered, and DNA of these cells was isolated by phenol extraction according to the method of Ivanovic *et al.* (31). Final DNA was dissolved at 1 mg/ml in 10 mM Tris·HCl, pH 7.4, and used for ^{32}P-postlabeling.

^{32}P-postlabeling of DNA adducts. The method of Randerath *et al.* (32) was followed, with modifications (25). Each experiment used equal amounts of DNA from different treatments (12 μg). A sample of calf thymus DNA alkylated by xanthine oxidase-activated MMC (15, 25) was labeled and prepared at the same time and was used as a standard.

TLC separation of ^{32}P-labeled adducts. Equal amounts of ^{32}P-labeled sample (0.5 μg of DNA) were spotted onto CN-HPTLC silica gel plates and analyzed by the two-dimensional system developed earlier (25). At the same time, a sample of standard was developed under the same conditions. Identification of radioactive spots separated from unknown samples was obtained by comparison with standard plate.

Quantitative estimation of radioactive spots separated by TLC. Radioactivity of each spot separated on TLC plates was measured with a Betascope 603 blot analyzer (βetagen, Waltham, MA). Background radioactivity was measured by taking three readings of blank areas of average spot size. Net radioactivity of each spot was obtained by subtracting the average of the three blank readings from readings of each radioactive spot.

Statistical analysis. Data were analyzed by paired *t* test and Wilcoxon test. The level of significance was $p < 0.05$.

Results

Stability of extracellular and intracellular pH of cells in HBSS media. A standard curve for BCECF measurements of intracellular pH in both cell lines was established. There was a linear relationship between intracellular pH (5.8–7.6) and the fluorescence ratio (f_1/f_2) of emission at 530 ± 10 nm and 630 ± 22 nm ($r^2 = 0.997$; Fig. 1). This standard curve is similar to those obtained with Chinese hamster ovary cells (26) and suggested that an equilibration of extra- and intracellular pH was achieved under the conditions used. After equilibration in HBSS media under conditions used for drug exposure, the intracellular pH measurement of HCT 116 cells exceeded extracellular pH by about 0.25 units at pH 6.0, 0.1 unit at pH 7.2, and 0.08 unit at pH 7.6 (Table 1). The pH of HBSS medium, within the range of 6.0–7.6 used, remained stable during the 120-min incubation with cells ($p < 0.05$). Intracellular pH remained relatively stable for 60 min of incubation in HBSS medium after equilibration (control cells, $p < 0.05$). The presence of MMC did not affect the intracellular pH of HCT 116 cells during drug exposure at pH 7.2 or 7.6 ($p < 0.05$) but

TABLE 1

Intracellular pH of HCT 116 cells measured by flow cytometric analysis

Cells (5×10^5/ml) were harvested, loaded with BCECF/AM, and resuspended in HBSS medium as described in Materials and Methods. After equilibration for 30 min, MMC at various concentrations or equivalent buffer for control cells was added. Extracellular pH was the pH of the HBSS medium, which was measured with a pH meter. Intracellular pH was measured as the fluorescence ratio (f_1/f_2) of the emission from BCECF at 530 ± 10 nm (f_1) and 630 ± 20 nm (f_2), with a FACStar. Both extracellular and intracellular pH measurements were performed immediately after the addition of MMC (60 min) and at the end of 60-min drug treatment (120 min). Extracellular pH data represent mean ± standard error of five determinations. Intracellular pH data represent mean ± standard error of two separate measurements of two experiments. with MMC at 12.8 μM.

Time	Extracellular pH		
60 min	5.98 ± 0.04	7.18 ± 0.05	7.62 ± 0.05
120 min	6.02 ± 0.04	7.22 ± 0.04	7.60 ± 0.04
Time	**Intracellular pH**		
Control cells			
60 min	6.32 ± 0.05	7.34 ± 0.05	7.63 ± 0.07
120 min	6.26 ± 0.07	7.33 ± 0.05	7.61 ± 0.10
MMC-treated cells			
60 min	6.26 ± 0.05	7.33 ± 0.04	7.68 ± 0.07
120 min	6.10 ± 0.06	7.35 ± 0.05	7.59 ± 0.09

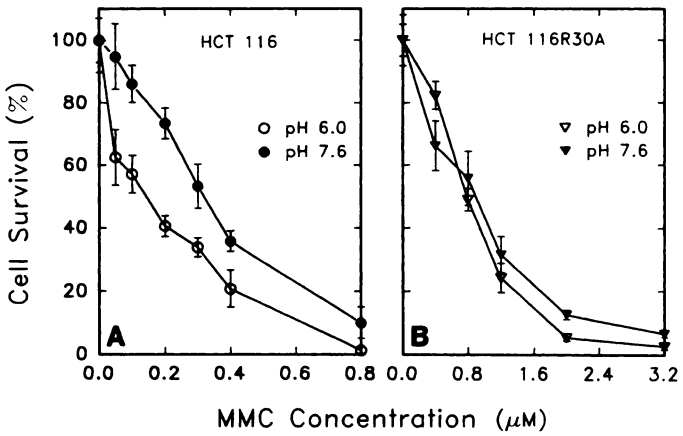

Fig. 2. Survival of HCT 116 and HCT 116-R30A cells with different intracellular pH values after 60-min exposure to MMC. Cells were equilibrated for 60 min in HBSS medium at the designated pH and were then exposed to various concentrations of MMC in the same medium for 60 min. Cells were then washed free of drug and cloned in McCoy's 5A complex medium. After incubation for 10 days at 37° with 5% CO_2 and 95% humidity, colonies with >50 cells were counted. The cloning efficiency for each treatment was determined and expressed as a percentage of control. Each *point* represents the mean ± standard error of at least four experiments. A, HCT 116 cells at pH 6.0 and 7.6; B, HCT 116-R30A cells at pH 6.0 and 7.6.

slightly affected the intracellular pH of HCT 116 cells during drug exposure at pH 6.0 ($p > 0.05$). All data obtained during the study of HCT 116-R30A cells were similar to those from the study of HCT 116 cells.

Effect of intracellular pH on MMC cytotoxicity. Under aerobic conditions, HCT 116 cells became more sensitive to MMC at a lower pH. The IC_{50} of MMC decreased from 0.3 ± 0.04 μM at pH 7.6 to 0.1 ± 0.03 μM at pH 6.0 ($p < 0.05$) (Fig. 2A). Survival of HCT 116 cells treated at pH 7.2 was between those of cells treated at pH 6.0 and 7.6. In contrast, changing intracellular pH from 7.6 to 7.2 or 6.0 did not affect the sensitivity of HCT 116-R30A cells to a 1-hr exposure to MMC. The IC_{50} of MMC at these three hydrogen ion concentrations was 1.0 μM, with little variation (Fig. 2B). There was an approximately 3-fold difference in IC_{50} values between the MMC-sensitive and the MMC-resistant cell lines in the current measurement, instead of 5-fold as reported earlier (24). This discrepancy was caused by the 2-hr exposure of cells in serum-free medium. During anaerobic exposure to MMC, no difference in cell survival was detected between the two cell lines, and the IC_{50} of MMC was 0.03 μM for both cell lines.

Effect of intracellular pH on DNA interstrand cross-linking. MMC induced interstrand DNA cross-linking in both cell lines (Fig. 3). The amount of cross-linking increased as drug concentration increased from 3.2 to 6.4 and 12.8 μM (data for 3.2 and 6.4 μM are not shown). The amount of interstrand DNA cross-linking in HCT 116 cells increased as the intracellular pH decreased from 7.6 to 6.0 (Fig. 3A). The difference between pH 7.6 and 6.0 was statistically significant ($p < 0.05$). The difference between pH 7.2 and 6.0 was not as significant ($p < 0.2$). The effect of intracellular pH of MMC-DNA interstrand cross-linking in the MMC-resistant HCT 116-R30A cells was less detectable (Fig. 3B). When the two cell lines were compared after treatment at the same intracellular pH, the amount of interstrand DNA cross-linking was always greater in HCT 116 cells than in HCT 116-R30A cells.

MMC-DNA adducts produced by MMC activated by cellular fractions. Depending on the pH value and oxygen tension, different adduct patterns were produced when calf thymus DNA interacted with MMC that had been activated by different cellular fractions of MMC-sensitive and MMC-resistant cells. The specifically identified adduct patterns resulting from the interaction of calf thymus DNA and xanthine oxidase-activated MMC (25) were used as standards. Minimal MMC adducts were detected by TLC when MMC was activated under aerobic conditions by microsomal fractions of sensitive or resistant cell lines at either pH 6.0 or 7.6 (data not shown). Under anaerobic conditions, activation of MMC by microsomes of both cell sources produced two different adduct patterns, de-

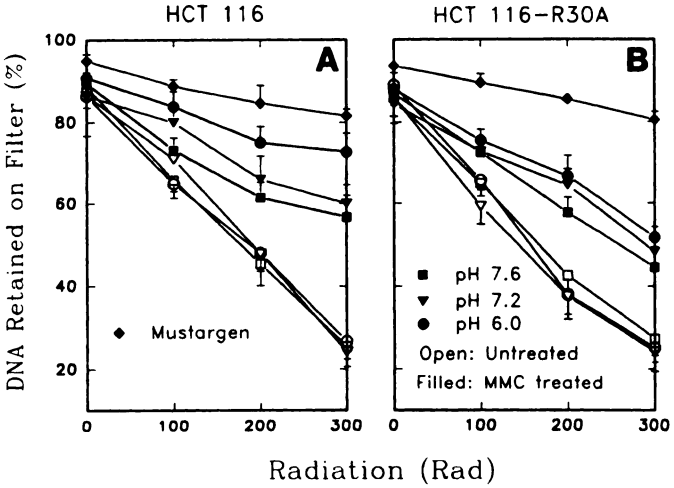

Fig. 3. MMC-induced DNA interstrand cross-linking in HCT 116 and HCT 116-R30A cells. Both types of cells were prelabeled with [^{14}C]thymidine and then exposed to 12.8 μM MMC in HBSS media at pH 6.0, 7.2, and 7.6 after a 60-min equilibration, as described in Materials and Methods. Cells were recovered, washed, and exposed to specified doses of X-irradiation, and alkaline elution was performed as described in Materials and Methods. Concomitant positive controls with 5 μM mustargen were included in each experiment. *Points* represent the mean ± standard error of two experiments, each performed in duplicate. Statistical analyses were conducted with data sets for pH 7.6 and 6.0 ($p < 0.05$) and for pH 7.2 and 6.0 ($p < 0.2$) by Wilcoxon test.

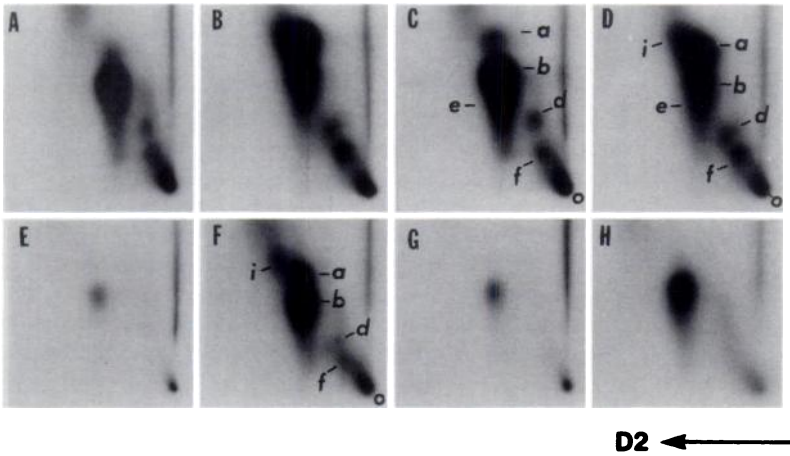

Fig. 4. Two-dimensional TLC analysis of ^{32}P-labeled nucleotides from DNA alkylated at pH 6.0 and pH 7.6 by MMC activated by microsomal and soluble fractions of HCT 116 and HCT 116-R30A cells. The preparation of MMC-DNA, the procedure for ^{32}P-labeling, and the two-dimensional TLC development were as described previously (25). Equal amounts of DNA (0.5 μg) from eight different treatments were spotted. *Top*, MMC activated by microsomes of HCT 116 cells at pH 7.6 (A), HCT 116 cells at pH 6.0 (B), HCT 116-R30A cells at pH 7.6 (C), and HCT 116-R30A cells at pH 6.0 (D). *Bottom*, MMC activated by soluble fractions of HCT 116 cells at pH 7.6 (E), HCT 116 cells at pH 6.0 (F), HCT 116-R30A cells at pH 7.6 (G), and HCT 116-R30A cells at pH 6.0 (H). *o*, origin.

pending on the pH of the reaction (Fig. 4, A–D). From DNA alkylated at pH 6.0, six different radioactive spots (**a**, **b**, **d**, **e**, **f**, and **i**) were separated by TLC (Fig. 4, B and D), whereas five spots (**a**, **b**, **d**, **e**, and **f**) were separated from the DNA alkylated at pH 7.6 (Fig. 4, A and C).

Identities of the spots were assigned based on comparison with adducts produced from concomitantly performed standard reactions of calf thymus DNA and xanthine oxidase-activated MMC (Table 2). Spots **a**, **b**, and **f** were identified as αN^2-G-MMC, N^2-G-DMC, and βN^2-G-MMC, respectively. Spot **e**, which was overshadowed by spot **b**, was identified as the cross-linked adduct $(N^2\text{-G})_2$-MMC. Spot **d** was not identified, and spot **i** was thought to be a nucleotide resistant to nuclease digestion. As we demonstrated previously (25), pH is the determining factor for types of adducts shown by the two TLC separation patterns. Analysis by Betascope of the relative intensity of radioactive spots further illustrated the differences in distribution ratios of these spots (Table 2).

Under aerobic conditions at pH 7.6, the soluble fraction of either HCT 116 or HCT 116-R30A cells activated MMC to alkylate calf thymus DNA and yield only one radioactive spot, spot **b** (Fig. 4, E–H). The radioactivities of the spots obtained were similar (Table 2). At pH 6.0, DNA alkylation by MMC activated by the soluble fraction of the MMC-sensitive cells produced three spots (**a**, **b**, and **f**) with assigned identities and two unidentified spots (**d** and **i**), an adduct pattern similar to that produced by microsomes at pH 6.0. In contrast, MMC activation by the soluble fraction of MMC-resistant cells at pH 6.0 produced only one spot, spot **b**. Under anaerobic conditions, interaction of calf thymus DNA with MMC activated by the soluble fraction of either cell line produced results similar to those produced under anaerobic conditions (data not shown).

Effect of intracellular pH on adduct formation in cellular DNA. Depending on the cell type and the intracellular pH during drug treatment, DNA isolated from cells exposed to 25 μM MMC showed different ^{32}P-postlabeling adduct patterns (Fig. 5). DNA from HCT 116-R30A cells exposed to MMC at pH 6.0 and 7.6 produced two similar radioactive spots (**b** and **f**) (Fig. 5, B and D). DNA from HCT 116 cells exposed to MMC at pH 6.0 and 7.6 produced two different TLC patterns. At pH 7.6, two spots (**b** and **f**), similar to those produced by DNA from the HCT 116-R30A cells, were resolved, although the intensity was somewhat greater (Fig. 5A). At pH 6.0, however, DNA from HCT 116 cells produced seven radioactive spots (**a**, **b**, **d**, **f**, **g**, **h**, and **i**) (Fig. 5C). Proportionally, a large amount of spot **f** was detected in cellular DNA from either cell line treated at either pH value. There were no radioactive spots from control cells of either type after incubation at pH 6.0 or 7.6 without exposure to MMC. Analysis by Betascope of the relative intensity of radioactive TLC spots further illustrated the difference in DNA from the two types of cells treated at pH 6.0 and 7.6 (Table 2).

By comparison with the standard, the identities of spots **a**, **b**, and **f** were tentatively assigned as N^2-G-DMC, $1''\alpha N^2$G-MMC, and $1''\beta N^2$-G-MMC, respectively. Spots **d** and **i** were observed previously in a study of the interaction of DNA with MMC activated by xanthine oxidase, where spot **d** was an unknown and spot **i** was thought to be a nucleotide resistant to nuclease digestion. The identities of two new radioactive spots, **g** and **h**, remain unknown.

Discussion

Acidic pH enhanced both drug response and MMC-induced DNA cross-linking in HCT 116 cells. Kennedy *et al.* (17) have reported similar observations in EMT6 cells. We have shown that the HCT 116 cells contain high levels of quinone reductase (24), and by assaying the reduction of 2,6-dichlorophenol-indophenol we have shown that EMT6 cells contained levels of quinone reductase similar to those in HCT 116 cells.[1] We feel that the high content of quinone reductase in these cells is responsible for the DNA cross-linking and aerobic cytotoxicity of MMC that can be further enhanced by acidic intracellular pH.

Using microsomes and soluble extracts separately to catalyze MMC activation and DNA adduct formation, we tested the action of NADPH-cytochrome P450 reductase and quinone reductase associated with these fractions. Our data show that MMC intermediates produced by soluble extracts have the ability to alkylate DNA. At pH 6.0, HCT 116 soluble extract was more active than HCT 116-R30A soluble extract. This is presumably due to the higher level of quinone reductase present in HCT 116 cells, because other reductive enzymes contained in microsomes and mitochondria were not present in the soluble fraction preparations used. The presence or absence of oxygen had no effect on the DNA adduct formation by MMC intermediates produced by soluble fractions. On the other hand,

[1] S-S. Pan, F. Y., and C. Hipsher unpublished observations.

TABLE 2

Distribution of ^{32}P-postlabeled DNA adducts in MMC-alkylated DNA

Methods for preparation of MMC-alkylated DNA through activation by microsomes or soluble extracts of HCT 116 or HCT 116-R30A cells, isolation of cellular DNA from cells treated with MMC, ^{32}P-postlabeling of DNA adducts, TLC separation of DNA adducts, and quantitative analysis of radioactive spots on TLC plates by Betascope were described in detail in the text. Quantification was based on equal amounts of DNA (0.5 μg) subjected to TLC analysis. Data were obtained from an average of repeated experiments. The variation between experiments was <15%.

Spot	Identity	Radioactivity			
		HCT 116		HCT 116-R30A	
		pH 7.6	pH 6.1	pH 7.6	pH 6.1
		dpm			
Microsomes					
a	N^2-G-DMC	3,914	15,244	4,498	15,361
b + e	α N^2-G-MMC (N^2-G)$_2$-MMC	17,376	9,034	18,646	9,521
d	Unknown	1,481	1,558	1,330	1,689
f	β N^2-G-MMC	2,240	2,507	1,915	2,657
i	Unknown		2,787		2,345
Total		25,011	31,137	27,918	31,573
Soluble extracts					
a	N^2-G-DMC		5,069		
b + e	α N^2-G-MMC (N^2-G)$_2$-MMC	1,437	10,196	1,726	6,382
d	Unknown		1,078		
f	β N^2-G-MMC		1,771		
I	Unknown		1,418		
Total		1,437	19,532	1,726	6,382
Cellular DNA					
a	N^2-G-DMC		1,680		
b	α N^2-G-MMC	770	948	418	620
d	Unknown		1,138		
f	β N^2-G-MMC	3,269	11,825	1,291	1,256
i	Unknown		974		
g + h	Unknown		1,323		
Total		4,039	17,888	1,709	1,885

when MMC intermediates were produced by microsomal enzymes anaerobic conditions were required for DNA alkylation. Again, the pH of the reaction was the determining factor in the distribution of DNA adducts, as indicated by ^{32}P-postlabeling results.

The bifunctional cross-linked adduct was not detected in cellular DNA by the ^{32}P-postlabeling assay, due to either its resistance to nuclease digestion or low concentration. Measurement of cellular DNA cross-linking by MMC had to rely on the alkaline elution method. Nevertheless, total DNA adduct formation in cells responded to intracellular pH changes and the activity of quinone reductase in the two cell lines studied. Only HCT 116 cells with intracellular pH in the acidic range generated large amounts of MMC-DNA adducts, with the typical distribution pattern produced by reductive enzymes at low pH. The high level of quinone reductase in HCT 116 cells is probably responsible for this aerobic DNA alkylation at acidic intracellular pH. The bifunctional monoadduct βN^2-G-MMC was produced at 10-fold higher levels in the MMC-sensitive cells at pH 6 than in the MMC-resistant cells. MMC-resistant cells did not express a 10-fold increase in resistance. It is likely that this adduct is not the major factor for cytotoxicity of MMC, instead, bifunctional cross-linking is more important for cytotoxicity.

MMC-DNA adducts and MMC-DNA cross-linking were detected in both types of cells when the intracellular pH was adjusted to 7.2–7.6, a pH range that was not optimal for quinone reductase. Other reductive enzymes, including cytosolic, microsomal, and mitochondrial enzymes, could be responsible for MMC reduction at this higher pH range. This possibility is supported by our earlier observations, in HCT 116 and HCT 116-R30A cells at physiological pH (7.4), that DNA alkylation by radioactive porfiromycin was not totally inhibited by 100 μM dicoumarol (24). This is also supported by the recent report that xanthine dehydrogenase has a pH profile for reducing MMC similar to that of quinone reductase, but extending more into the physiological pH range (34). More DNA cross-linking and DNA adducts were detected in HCT 116 cells than in HCT 116-R30A cells at pH 7.6, which is an optimal pH for NADPH-cytochrome P450 reductase. Although the contents of NADPH-cytochrome P450 reductase in both cell lines are similar (24), the contents of other reductive enzymes have yet to be analyzed. Another possibility is that localized areas in cells may be under acidic conditions. The conditions used in the present study to obtain the desired intracellular pH were designed to provide a stable controlled environment. The pH-sensitive dye BCECF, used for flow cytometric analysis, is reported to be localized almost exclusively in the cytoplasm (33). Whether the pH is uniform through the whole cell, including the nucleus and mitochondria, under our experimental conditions is not known. The ultimate proof relating reductive activation of MMC by quinone reductase, over a wide pH range, to MMC-DNA adduct formation will require experiments with purified enzymes from both cell lines. These studies are in progress.

This study was conducted in a controlled environment. However, data obtained strongly suggest that the high level of quinone reductase in HCT 116 cells is responsible for the aerobic cytotoxicity of MMC that can be enhanced by acidic intracellular pH. Decreased activity of quinone reductase was used by MMC-resistant HCT 116-R30A cells as a mechanism

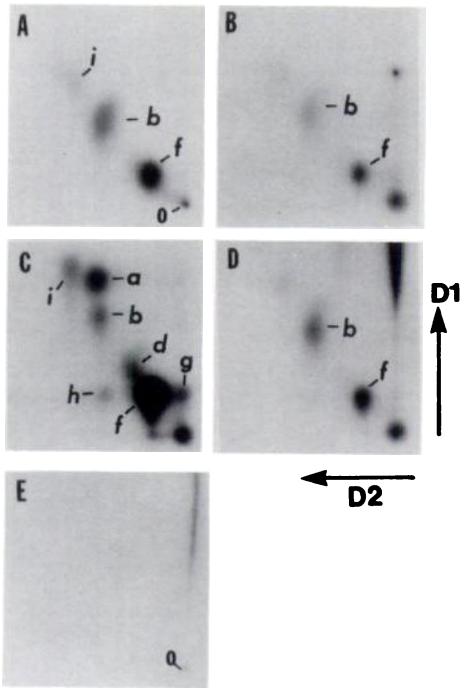

Fig. 5. Two-dimensional TLC analysis of ^{32}P-labeled nucleotides from DNA isolated from HCT 116 and HCT 116-R30A cells exposed to 25 μM MMC for 1 hr, with intracellular pH equilibrated to 6.0 and 7.6. The isolation of cellular DNA, the procedure for ^{32}P-labeling, and the two-dimensional TLC development were as described in the text. Equal amounts of DNA (0.5 μg) from cells from five sources were spotted. A and B, Cells treated at intracellular pH 7.6, HCT 116 (A) and HCT 116-R30A (B). C–E, Cells treated at intracellular pH 6.0; HCT 116 (C), HCT 116-R30A (D), and HCT 116 without MMC exposure (E). *O*, origin.

to avoid MMC-induced DNA damage under aerobic conditions. Bioreductive activation of MMC is one of the key factors in the multiple antitumor actions of this agent. NADPH-cytochrome P450 reductase, with a higher optimal pH range, has been thought to be the major enzyme for the activation of MMC. The demonstration that quinone reductase activates MMC in a low pH range, alkylating cellular DNA under both hypoxic and aerobic conditions, indicates a wider spectrum for MMC to function in solid tumors, which may contain a range of pH and oxygen concentrations. In an *in vivo* situation, to produce optimal metabolic and environmental conditions for solid tumors, containing a heterogeneous population of cells, that would favor the activation of MMC by any particular enzyme would be difficult. However, the wide range of enzymes, including NADPH-cytochrome P450 reductase (4), xanthine oxidase (4), xanthine dehydrogenase (34), cytochrome *b* reductase (35), quinone reductase (11, 13), and other reductases, provides enzymatic reductive capabilities for MMC activation over a wide span of conditions. Further understanding of the regulation of quinone reductase in tumor cells and of their ability to reduce various quinone agents should be helpful for future clinical applications of quinone-containing agents.

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References

1. Iyer, V. N., and W. Szybalski. Mitomycins and porfiromycin: chemical mechanism of activation and cross-linking of DNA. *Science (Washington D. C.)* **145**:55–58 (1964).
2. Moore, H. W. Bioactivation as a model for drug design bioreductive alkylation. *Science (Washington D. C.)* **197**:527–532 (1977).
3. Tomasz, M., and R. Lipman. Reductive metabolism and alkylating activity of mitomycin C induced by rat liver microsomes. *Biochemistry* **20**:5056–5061 (1981).
4. Pan, S., P. Andrews, C. Glover, and N. R. Bachur. Reductive activation of mitomycin C and mitomycin C metabolites catalyzed by NADPH cytochrome P-450 reductase and xanthine oxidase. *J. Biol. Chem.* **259**:959–966 (1984).
5. Peterson, D. W., and J. Fisher. Autocatalytic quinone methide formation from mitomycin C. *Biochemistry* **25**:4077–4084 (1986).
6. Doroshow, J. H. Mitomycin C-enhanced superoxide and hydrogen peroxide formation in rat heart. *J. Pharmacol. Exp. Ther.* **218**:206–211 (1981).
7. Kalyanaraman, B. E., E. Perez-Reyes, and R. R. Mason. Spin-trapping and direct electron spin resonance investigation of the redox metabolism of quinone anticancer drugs. *Biochem. Biophys. Acta* **630**:119–130 (1980).
8. Pritsos, C. A., and A. C. Sartorelli. Generation of reactive oxygen radicals through bioactivation of mitomycin antibiotics. *Cancer Res.* **46**:3528–3532 (1986).
9. Kennedy, K. A., S. Rockwell, and A. C. Sartorelli. Preferential activation of mitomycin C to cytotoxic metabolites by hypoxic tumor cells. *Cancer Res.* **40**:2356–2360 (1980).
10. Keyes, S. R., S. Rockwell, and A. C. Sartorelli. Porfiromycin as a bioreductive alkylating agent with selective toxicity to hypoxic EMT6 rumor cells *in vivo* and *in vitro*. *Cancer Res.* **45**:3642–3645 (1985).
11. Siegel, D., N. W. Gibson, P. C. Preusch, and D. Ross. Metabolism of mitomycin C by DT-diaphorase: role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res.* **50**:7483–7489 (1990).
12. Prakash, A. S., D. Ross, and N. W. Gibson. Sequence selective alkylation of DNA by mitomycin C (MMC) after reduction by DT-diaphorase (DTD). *Proc Am. Assoc. Cancer Res.* **33**:5 (1992).
13. Siegel, D., N. W. Gibson, and D. Ross. DT-diaphorase mediated activation of mitomycin C to DNA cross-linking species dependent on pH. *Proc. Am. Assoc. Cancer Res.* **33**:404 (1992).
14. Andrews, P. A., S. Pan, and N. R. Bachur. Electrochemical reductive activation of mitomycin C. *J. Am. Chem. Soc.* **108**:4158–4166 (1986).
15. Pan, S., T. Iracki, and N. R. Bachur. DNA alkylation by enzyme-activated mitomycin C. *Mol. Pharmacol.* **29**:622–628 (1986).
16. Pan, S., and T. Iracki. Metabolites and DNA adduct formation from flavoenzyme-activated porfiromycin. *Mol. Pharmacol.* **34**:223–228 (1988).
17. Kennedy, K. A., J. D. Mcgurl, L. Leondaridis, and O. Alabaster. pH dependence of mitomycin-induced cross-linking activity of EMT6 tumor cells. *Cancer Res.* **45**:3541–3547 (1985).
18. Keyes, S. R., S. Rockwell, and A. C. Sartorelli. Enhancement of mitomycin C cytotoxicity to hypoxic tumor cells by dicumarol *in vivo* and *in vitro*. *Cancer Res.* **45**:213–216 (1985).
19. Rockwell, S., S. R. Keyes, and A. C. Sartorelli. Modulation of the cytotoxicity of mitomycin C to EMT6 mouse mammary tumor cells by dicumarol *in vitro*. *Cancer Res.* **48**:5471–5474 (1988).
20. Marshall, R. S., M. C. Paterson, and A. M. Rauth. Deficient activation by a human cell strain leads to mitomycin resistance under aerobic but not hypoxic conditions. *Br. J. Cancer* **59**:341–346 (1989).
21. Marshall, R. S., M. C. Paterson, and A. M. Rauth. DT-diaphorase activity and mitomycin C sensitivity in non-transformed cell strains derived from members of a cancer-prone family. *Carcinogenesis (Lond.)* **12**:1175–1180 (1991).
22. Dulharty, A. M., and G. F. Whitmore. Chinese hamster ovary cell lines resistant to mitomycin C under aerobic but not hypoxic conditions are deficient in DT-diaphorase. *Cancer Res.* **51**:1860–1865 91991).
23. Begleiter, A., E. Robotham, G. Lacey, and M. K. Leith. Increased sensitivity of quinone resistant cells to mitomycin C. *Cancer Lett.* **45**:173–176 (1989).
24. Pan, S., S. A. Akman, G. L. Forrest, C. Hipsher, and R. Johnson. The role of NAD(P)H:quinone oxidoreductase in mitomycin C and porfiromycin resistant human colon cancer HCT 116 cells. *Cancer Chemother. Pharmacol.*, **31**:21–31 (1992).
25. Yu, F., and S.-s. Pan. Effect of pH on DNA alkylation by enzyme-activated mitomycin C and porfiromycin. *Mol. Pharmacol.* **43**:863–869 (1993).
26. Wang, Z., G. L. Chu, W. C. Hyun, H. A. Pershadsingh, M. J. Fulwyler, and W. C. Dewey. Comparison of DMO and flow cytometric methods for measuring intracellular pH and the effect of hyperthermia on the transmembrane pH gradient. *Cytometry* **11**:617–623 (1990).
27. Chou, J., and T. C. Chou. *Dose Effect Analysis with Microcomputers*. Elsevier Science Publishers, BV, Amsterdam, The Netherlands (1985).
28. Kohn, K. W., L. C. Erickson, R. A. G. Ewig, and C. A. Friedman. Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry* **15**:4629–4637 (1976).
29. Hilton, J. Deoxyribonucleic acid crosslinking by 4-hydroperoxycyclophosphamide in cyclophosphamide-sensitive and -resistant L1210 cells. *Biochem. Pharmacol.* **33**:1867–1872 (1984).
30. Cohn, N. A., M. J. Egorin, S. W. Snyder, B. Ashar, B. E. Wietharn, S. Pan, D. Ross, and J. Hilton. Interaction of *N,N′,N″*-triethylenethiophosphoramide and *N,N′,N″*-triethylenephosphoramide with cellular DNA. *Cancer Res.* **51**:4360–4366 (1991).
31. Ivanovic, V., N. E. Geacimtov, and I. B. Weinstein. Cellular binding of

benzo(*a*)pyrene to DNA characterized by low temperature fluorescence. *Biochem. Biophys. Res. Commun.* **70**:1172–1197 (1976).
32. Randerath, K., E. Randerath, T. F. Danna, K. L. van Golen, and K. L. Putman. A new sensitive ^{32}P-postlabeling assay based on the specific enzymatic conversion of bulky DNA lesions to radiolabeled dinucleotides and nucleoside 5′-monophosphates. *Carcinogenesis (Lond.)* **10**:1231–1239 (1989).
33. Paradisio, A. M., R. Y. Tsien, and T. E. Machen. Na^+-H^+ exchange in gastric glands as measured with cytoplasmic-trapped fluorescent pH indicator. *Proc. Natl. Acad. Sci. USA* **81**:7436–7440 (1984).
34. Gustafson, D. L., and C. A. Pritsos. Bioactivation of mitomycin C by xanthine dehydrogenase from EMT6 mouse mammary carcinoma tumors. *J. Natl. Cancer Inst.* **84**:1180–1185 (1992).
35. Hodnick, W. F., and A. C. Sartorelli. Reductive activation of mitomycin C by NADH cytochrome *b* reductase. *Proc. Am. Assoc. Cancer Res.* **32**:397 (1991).

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