A Bioassay to Measure Cytotoxicity of Plasma from Patients Treated with Mitomycin C

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ABSTRACT

The unpredictable clinical toxicity observed in patients treated with mitomycin C and the observation that this agent must be reduced to an active form before alkylating target molecules have led to the development of a bioassay which is capable of detecting biologically active forms of mitomycin C in the plasma of drug-treated patients. The bioassay makes use of a repair-deficient mutant of Chinese hamster ovary cells, UV-20, which is 40 to 60 times more sensitive to mitomycin C than its wild-type parent. A standard curve relating in vitro cell colony-forming ability of UV-20 versus drug concentration in the plating medium has been determined. Mitomycin C levels in patient plasma as low as 1 ng/ml can be detected, compared to the 5-ng/ml limit of detection obtained with a high-pressure liquid chromatography assay for the parent compound. This assay has been utilized to detect active drug in plasma obtained from patients with colorectal cancer treated with mitomycin C as a single agent. At the completion of drug injection, serial blood samples were collected in heparinized tubes, and aliquots of plasma were extracted and assayed for mitomycin C levels by high-pressure liquid chromatography, diluted and assayed directly for their toxicity for UV-20 cells, or frozen at -20°C to be assayed at a later time. The activity detected by immediate bioassy was stable up to 2 mo in frozen samples. Plasma pharmacokinetics determined by the bioassay in seven patients were no different than those determined by high-pressure liquid chromatography. No stable, cytotoxic species other than the parent compound were detected by the bioassay in the plasma of patients treated with mitomycin C.

INTRODUCTION

Mitomycin (MMC) has been referred to as the prototype of a class of chemotherapeutic compounds known as bioreductive alkylating agents (1). The parent MMC molecule is not biologically active. Cytotoxic activity requires its enzymatic reduction to a semiquinone (2) and/or a dihydroquinone (3), with subsequent elimination of the methoxy group and aziridine ring opening. This process produces an activated molecule capable of forming monoadducts and cross-links in DNA (4). Various chemical (2) and cell-free microsomal reduction systems (3) have been utilized to study the chemistry of mitomycin C and have identified several stable reduction products. The spectrum of products seen can vary from one reduction system to another, and it is not known which model system best represents human metabolism of MMC.

While clinically useful in the treatment of carcinomas of the breast, colon, stomach, and bladder, unpredictable toxicities which occasionally accompany MMC administration have limited its widespread use (5). It is not clear if these toxicities are associated with differences in the pharmacokinetics of parent MMC and its metabolites in individual patients. Recently, the use of HPLC techniques has resulted in comprehensive pharmacokinetic studies of MMC in patients which have suggested that some metabolites of MMC are present in the plasma of patients. Techniques have been now developed for measuring many of the metabolites of mitomycin C produced in model systems (6) and could be applied to patient samples. Little is known, however, about the cytotoxicity of these reduction products and what, if any, role they might play in the variable toxicity of the drug in individual patients.

One investigative approach into the possible biological role of such metabolites is the use of a bioassay to determine whether possible toxic metabolites of MMC are present in the plasma of patients undergoing chemotherapy. If toxicity other than that due to the parent itself were observed, the chemical nature of the active metabolite(s) could be investigated, and correlations could be sought with observed patient to patient variations in drug toxicity. This in turn would help to focus attention on which metabolites are of greatest biological interest. In fact, early studies of the pharmacology of MMC utilized a bioassay in which Escherichia coli B was used as an indicator for MMC activity, but only limited data were reported (7).

In the present work, a bioassay has been developed which incorporates a mutant Chinese hamster ovary cell line which is extremely sensitive to MMC. This assay is used to determine the activity of MMC and its possible metabolites in human plasma samples. Comparison of this assay to an HPLC assay has been made to determine the degree to which parent MMC levels in plasma can account for the activity observed in the bioassay.

MATERIALS AND METHODS

Cells. The cells utilized in these experiments were CHO wild-type (AA8-4) and a repair-deficient mutant (UV-20) derived from it. The UV-20 mutant is known to have increased sensitivity to UV light and MMC (7, 8). While clinically useful in the treatment of carcinomas of the breast, colon, stomach, and bladder, unpredictable toxicities which occasionally accompany MMC administration have limited its widespread use (5). It is not clear if these toxicities are associated with differences in the pharmacokinetics of parent MMC and its metabolites in individual patients. Recently, the use of HPLC techniques has resulted in comprehensive pharmacokinetic studies of MMC in patients which have suggested that some metabolites of MMC are present in the plasma of patients. Techniques have been now developed for measuring many of the metabolites of mitomycin C produced in model systems (6) and could be applied to patient samples. Little is known, however, about the cytotoxicity of these reduction products and what, if any, role they might play in the variable toxicity of the drug in individual patients.

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BIOASSAY FOR MMC

Chemicals. Mutamycin (Bristol-Myers, Ottawa, Canada) containing 5 mg of drug and 10 mg of mannitol was reconstituted with 10 ml of sterile, deionized water prior to dilution into growth medium to give the desired concentration for cell exposure. The stock drug was made up immediately before use or stored at 500 µg/ml at 4°C for no longer than 2 wk before use. The same source and formulation of drug were used for the in vitro cell experiments as were used for patient chemotherapy.

Patient Samples. Blood samples were obtained from consenting patients with metastatic or locally recurrent carcinoma of the colon or rectum. One blood sample was taken from each patient immediately prior to MMC administration. All patients from whom blood samples were taken were received 15 to 25 mg of MMC i.v. through the side arm of a free-running i.v. over a period of 10 min. Samples were collected through a heparin lock from the arm opposite to which the drug was administered as a function of time after completion of the infusion. The heparinized blood was kept on ice until centrifuged at 250 × g for 10 min at 4°C. Plasma aliquots were removed under sterile conditions and either assayed immediately or frozen at −20°C for future analysis.

HPLC Assay. The level of MMC in patient plasma samples was assayed by HPLC according to the method of den Hartigh et al. (10). Briefly, MMC was extracted by mixing 0.2 ml of plasma sample with 1.8 ml of a chloroform:2-propanol mixture (1:1, wt/wt). After vortexing for 1 min and centrifuging for 5 min at 1000 × g, the clear supernatant was removed and evaporated to dryness at 37°C. The residue was then dissolved in 100 µl of methanol, and aliquots were injected into a Waters HPLC system with a C18 µBondapak reverse-phase column and a mobile phase of 35% methanol:65% water at a flow rate of 1 ml/min. The effluent was monitored at 268 nm, the absorption maximum of the parent molecule, and MMC was observed to elute at 7 min. The efficiency of the extraction procedure for plasma was 78 ± 9% (n = 14) as determined by HPLC according to the method of den Hartigh et al. (10).

Bioassay. This assay of MMC did not require an initial extraction of patient plasma. The pre- and post-drug addition plasma samples were diluted directly through a series of 3-fold dilutions in α-MEM medium. One- to 0.1-ml samples were added to 60-mm Petri dishes containing 5 ml of growth medium, each point being repeated in triplicate. One hundred UV-20 cells were then added to each dish and then incubated in a humidified 5% CO2:95% air atmosphere in a 37°C incubator. Colony-forming ability resulting from continuous exposure of cells to the MMC-containing plasma was assessed after 8 days. Continuous exposures were utilized to maximize the assay’s ability to detect biological activity in plasma and also avoid further manipulations which would disturb cell attachment to the dishes. Control cell-plating efficiency was 0.9 or higher. Utilizing a standard curve of cell survival after continuous exposure to MMC, mitomycin C equivalents present during incubation were determined. Comparison of bioassay and HPLC assay results allowed determination of cytotoxicity beyond that which could be attributed to parent compound alone. Stable cytotoxic metabolites present in plasma or stable substances in patient plasma which interfered with toxicity would produce MMC levels determined by bioassay which were different from those measured by HPLC.

Data Analysis. Analysis of patient data was conducted with the aid of a BMD® nonlinear curve-fitting program, and half-lives of the distribution and elimination phases of the drug in plasma were determined. The pharmacokinetic parameters presented in Table 1 were calculated for instantaneous drug injection utilizing conventional means (11). Student's t test was utilized to compare mean values obtained by HPLC and bioassay.

RESULTS

Initial experiments were done to document the relative sensitivity of CHO wild-type cells (AA8-4) and the repair-deficient mutant (UV-20) derived from it (8). Previous experiments had indicated a 70- to 80-fold increase in sensitivity of UV-20 to MMC under conditions of acute (8) or continuous (9) exposure. In the present experiments, 100 to 108 cells per dish were exposed continuously to MMC in growth medium during incubation for cell colony-forming ability. No evidence for any effect of cell density on toxicity was observed over this range of cell densities. The results shown in Chart 1 indicate that UV-20 (open symbols) has a 40- to 60-fold increase in sensitivity to MMC compared to wild-type cells (closed symbols). Sensitivity is observed at drug concentrations as low as 0.3 to 0.5 ng/ml and has been reproduced in four separate experiments, only two of which, for reasons of clarity, are presented in Chart 1. Since the peak plasma levels of drug in patients receiving MMC chemotherapy...
are 1 to 2 μg/ml (12–14). UV-20 appeared to be a particularly suitable target cell for bioassay of mitomycin C activity in human plasma.

By replotting the data for UV-20 shown in Chart 1 along with data from two other experiments on a linear-linear scale of the percentage of survival versus drug concentration, the results (solid symbols) shown in Chart 2 were obtained. The data were fit with a straight line which passes through 100% survival at zero drug concentration. In order to use such results for a calibration curve of MMC activity, it was necessary to determine if human plasma itself affected the assay. Known concentrations of MMC were added to plasma obtained from untreated human volunteers, and the plasma was diluted and added to UV-20 cells for continuous exposure during colony formation. The results are shown in Chart 2 (open symbols). As can be seen, addition of MMC to plasma before assay had no effect on the toxicity of drug towards UV-20 cells. In the course of these experiments it was observed that drug-free plasma concentrations in the colony assay greater than 20% decreased cell-plating efficiency. Heat inactivation of the plasma (56°C for 0.5 h) decreased this toxicity but also decreased MMC levels, and hence, it was not routinely incorporated. Therefore, all subsequent assays were carried out at levels of plasma in the assay dishes below 20%. Chart 2 was used as a standard curve for relating plasma toxicity to drug concentration in the following studies. For example, a reduction of survival to 50%, corresponded to a MMC level in the assay plate of 0.47 ng/ml.

A series of initial tests were done to determine how stable the MMC activity was in patient plasma as a function of handling after blood collection. Plasma samples which were immediately frozen after collection and held for up to 2 mo at -20°C had the same activity in either assay as fresh plasma assayed immediately after collection. Blood samples could also be held on ice for up to 6 h before assay with no loss of activity. However, if blood samples were left at room temperature for 18 h, no activity was detected in plasma. It was also noted that samples, which were initially frozen and thawed and then refrozen, lost activity in both assays on each subsequent freezing and thawing cycle. Therefore, samples were routinely collected on ice and immediately assayed, or the plasma was frozen and assayed within 2 mo with only a single freeze-thaw cycle.

For all patients, the control point of the bioassay was obtained by adding a sample of plasma obtained immediately prior to drug injection. The final volume of plasma in the assay dish was always less than 20%. In no case was any toxicity observed to be due to this predrug sample, plating efficiencies remaining at 0.9 or higher.

In monitoring the HLPC effluent at 365 nm, it was observed that, up to 4 h after infusion, only one absorption peak due to parent compound was present. Two other peaks which eluted at 9.2 and 10.4 min were detected in some samples collected at 5 and 6 h. Plasma samples taken immediately before administration of the drug did not show any absorbing peaks.

A comparison of the MMC concentrations determined by HPLC and by the bioassay was performed on samples obtained from patients receiving MMC as a single agent. Typical results for one patient are shown in Chart 3 for HPLC assay (solid symbols) and the bioassay (open symbols). The two assays were in close agreement. Table 1 summarizes the coefficients and plasma half-lives determined by a biexponential fit of the data points for bioassay and HPLC assay. There was not a significant difference between the results obtained by the two assays as determined by Student's t test (P > 0.05).

A graphic comparison of individual bioassay and HPLC determinations of plasma MMC activity and MMC concentrations, respectively, is given in Chart 4. The data could be best fit by a line of slope 0.86 ± 0.08 with the the line of Slope 1 lying within the 95% confidence interval. The correlation coefficient of the two techniques was 0.98.

DISCUSSION

The UV-20 cell line has proven to be a suitable system for assaying the biological activity of MMC in human plasma. The
assay developed appeared reliable, reproducible, and accurate. It routinely detected activity equivalent to parent drug at levels as low as 1 ng/ml. As a result, patient plasma could be examined for the presence of activity at periods up to 6 h after the completion of drug infusion. In addition, in this group of patients, the pre-drug plasma samples were without toxicity. This is, perhaps, not surprising since these patients were receiving MMC as single-agent chemotherapy. It was therefore not necessary to correct for background plasma toxicity.

The peak levels and half-lives of parent MMC obtained in the HPLC assay are similar to data we have collected in a study of 18 patients in which HPLC data alone were collected (submitted) and reports of others using a similar HPLC assay (12, 15). While the sensitivity of the HPLC assay used was reported to be 1 ng/ml (10), levels below 5 ng/ml could not be detected consistently in our studies. Thus, the sensitivity of the bioassay was better than that obtainable in the HPLC assay for parent MMC in the present work. What is more important, however, is that both assays were sensitive enough to monitor drug levels up to 6 h after drug injection, increasing the possibility of detecting differences which would indicate the presence of toxic metabolites.

Combining the data from the bioassay, which measures biological activity in the plasma in terms of MMC equivalents of toxicity, with the data from the HPLC assay, which detects parent MMC, it appears that cytotoxicity was directly due to the amount of parent MMC present. There was no evidence to suggest the possibility that there was a stable cytotoxic metabolite which would be evidenced by the clustering of points above the theoretical line of equivalence in Chart 4. Since the bioassay uses the patient plasma directly, potentially toxic metabolites were not lost during an extraction procedure.

No activity other than that attributable to MMC itself was detected at any time in the patients tested. Specifically, Patient 5, the only patient of this series to develop a clinically manifested toxicity, did not show any indication of detectable cytotoxic metabolites. The same was true for samples collected at 5 h after drug infusion, where low levels of unidentified metabolites were observed. It will be necessary, however, to examine the plasma of a larger number of patients which subsequently develop toxicity as a result of treatment to substantiate this observation. The present work is consistent with MMC activation proceeding through a short-lived semiquinone radical or dihydroquinone which spontaneously decomposes to other short-lived intermediates with the latter quickly reacting with nucleophiles, such as DNA and other macromolecules. Such intermediates would not be detected in the present bioassay.

The failure of the HPLC assay to detect appreciable levels of metabolites of MMC in the plasma may be due to the procedure used. The HPLC assay involves extraction of the plasma sample, and it is possible that the procedure utilized does not extract the metabolites. It is also possible that metabolites are retained on the column and are never eluted or that they absorb at wavelengths other than those which were monitored. Analysis of electrolytically reduced products of MMC by the present HPLC assay demonstrated retention of the reduced drug on the column (data not shown). A more extensive HPLC procedure recently developed by Andrews et al. (6) may permit more definitive testing for the presence of MMC metabolites in human plasma. The present bioassay result, therefore, does not rule out the presence of such metabolites. It does, however, indicate that they will have limited toxicity towards UV-20 cells in comparison to that of the parent MCC present in the plasma.

A bioassay for mitomycin C activity reported previously (7) utilized zones of growth inhibition of E. coli on agar plates to determine drug concentration. The minimum assayable concentration of MMC in solution was given as 2 ng/ml, but it was not stated whether this level of sensitivity could be obtained using human plasma. Also, at that time, no HPLC assay was available with which to compare the bioassay in terms of amount of parent drug or metabolites present. In the present work, the use of mutant mammalian cells rather than bacteria as indicators allowed tests of toxicity to be conducted with a system which was more closely related to that of human cells.

The strength of a bioassay is that it allows the biological activity present in plasma to be measured. This, combined with a chromatographic assay which identifies and quantitates the drug species present, can allow important drug metabolites to be identified. Routine monitoring of patient plasma for such metabolites with a more rapid HPLC assay could then be carried out, and adjustments to chemotherapeutic treatment schedules could be made with a rational basis. The current bioassay, though it failed to identify a toxic species other than parent MMC, may be useful for monitoring the biological activity of other chemotherapeutic agents to which the target cell is sensitive. For example, UV-20 has a 70-fold increase in sensitivity to cisplatinum compared to its wild-type parent AA8 (16). Thus, the current assay could be used for monitoring the biological activity of this and other adduct-forming agents as well. Combining the bioassay with chromatographic assays for these drugs and their metabolites may allow the identification of metabolites important to their effectiveness and toxicity.

REFERENCES

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