Pharmacodynamic Evaluation of Mitomycin C Analog BMS-181174 for Potential use in Intravesical Bladder Cancer Therapy

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INTRODUCTION

Our laboratory has been investigating approaches to improve the treatment efficacy of bladder cancer by intravesical therapy. Our previous studies have shown that (a) the variable and incomplete response of superficial bladder cancer patients to intravesical mitomycin C (MMC) therapy is in part due to the low chemosensitivity of some tumors and in part due to the inability to deliver sufficient drug concentrations to the tumor site, and (b) invasive tumors are less sensitive to MMC than superficial tumors (1,2). Logical approaches to improve the therapeutic efficacy of intravesical bladder cancer chemotherapy include enhancing the drug delivery to tumors and identifying more effective/potent drugs. The merit of pharmacokinetic interventions that aim to increase the MMC concentration at the target site is being evaluated in an ongoing phase III clinical trial in superficial bladder cancer patients (3). The present study focused on identifying new effective drugs.

MMC is an alkylating agent and is activated by intracellular quinone reductases. The two key activating enzymes are NADPH cytochrome p450 reductase and DT diaphorase, and their suppression has been linked to emergence of drug resistance (4–6). A new MMC analogue, N7-[2-(nitrophenylthio)ethyl] MMC or BMS-181174 (BMS, previously BMY-25067), has shown 10–200 fold greater cytotoxicity against several rodent and human tumor cell lines and has no cross-resistance with MMC (7–11). The reductive activation of BMS is independent of NADPH cytochrome p450 reductase and DT diaphorase activities and appears to involve non-enzymatic thiol-induced activation (12,13). Contrary to MMC which has a higher activity in hypoxia, BMS is equally effective under aerobic and hypoxic conditions (13). Preclinical toxicology studies indicate that this analog has a lower hematological toxicity than MMC and little cardiac, renal and pulmonary toxicity (7,14).

To determine if BMS may be a useful alternative to MMC for intravesical therapy, the potency and the kinetics of cytotoxicity of MMC and BMS were compared. Based on our previous finding of different MMC activities in 2-dimensional monolayer and 3-dimensional multilayer cultures (15), studies were performed using both culture systems to determine if the previously observed differential activities of the two drugs in monolayers (7–10) also occurred in multilayers. Two bladder tumor cell lines RT4 and TCC-SUP were used. RT4 cells were derived from a superficial bladder tumor, and TCC-SUP cells were isolated from an anaplastic transitional cell carcinoma in the neck of the urinary bladder (16). In vitro cytotoxicity indicates that RT4 is a non-invasive, well-differentiated papillary epidermoid carcinoma consistent with urinary bladder primary tumor grade I, whereas TCC-SUP is an invasive, high grade transitional carcinoma (17).

MATERIAL AND METHODS

Chemicals and Supplies

Sterile pigskin collagen (Spongostan Standard) was purchased from Health Designs Industries (Rochester, NY). RT4 and TCC-SUP cells from American Type Culture Collection (Rockville, MD), and tissue culture media from Gibco Laboratories (Grand Island, NY). MMC and BMS were gifts from Bristol Myers Squibb Co. (Wallingford, CT). Bromodeoxyuridine (BrdU) and its antibody were from Sigma Chemicals Co. (St. Louis, MO) and Biogenic (San Ramon, CA), respectively. All other-chemicals were of HPLC or cell culture grade and were used as received.

Cell Culture

RT4 cells were maintained in McCoy's medium supplemented with 9% heat-inactivated fetal bovine serum, 0.1 mM non-essential amino acids, 100 μg/ml gentamicin and 95 μg/ml cefotaxime. TCC-SUP cells were maintained in minimal essential medium (MEM) containing 0.1 mM sodium pyruvate. All other supplements were the same as for the RT4 medium. The pH of the media was adjusted to 7.4 with 0.1 mM NaOH. Both cell lines were grown to 90% confluence in T-150 flasks in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cells were then harvested by washing twice with calcium- and magnesium-free Hank's balanced salt solution containing 0.016% of EDTA and incubating for 3 to 5 min in 0.05% trypsin-EDTA. Thereafter, cell pellets were obtained by centrifugation at 1,000 X g for 10 min. For monolayer cultures, cells were resuspended in fresh medium seeded in 96-well plates (10,000 RT4 cells and 7,000 TCC-SUP cells per well) and grown for 24 hr. For multilayer cultures, cell pellets containing about 10⁶ cells were pipetted onto a piece of collagen gel (1 cm²) and were cultured for 48 hr prior to the experiment. The multilayer cell pellet consisted of 300 to 450 cell layers and was about 4 to 5 mm in diameter.

Pharmacodynamic Studies

On the day of experimentation, MMC was dissolved in sterilized water and BMS in 50:50 (v/v) ethanol:water. The stock solutions were dissolved in the media to give a final
concentration of 0.01 to 2.000 μg/ml, corresponding to 0.03 to 5.830 μM for MMC and 0.018 to 3.652 μM for BMS. The final concentration of ethanol in culture medium was <0.1%. All experiments with BMS, because of its sensitivity to light, were conducted under yellow light (400 nm). The experiment was initiated by replacing the maintenance culture medium with drug-containing medium, 4 ml for monolayers and 0.2 ml for multilayers in 6-well plates and 0.2 ml for monolayers in 96-well plates. The duration of drug treatment was 2 hr, which is equivalent to the duration of drug instillation during intravesical therapy in patients. After treatment, the cultures were washed 3 times with drug-free medium. For monolayers, cells were fixed, immediately or after 12, 24 and 48 hr incubation in drug-free medium, in 10% trichloroacetic acid at 4°C for 1 hr, rinsed with tap water 5 times, and stained with 0.4% sulforhodamine B (SRB) for 10 min. SRB stains for nuclear protein (18) and therefore provides an indirect measurement of cell numbers. After washing off the excess SRB with 1% acetic acid, the plates were air-dried overnight, and the protein-bound SRB was dissolved in 10 mM Tris HCl, pH 6. The optical density at 490 nm was determined using a plate reader (Bio-TEK Instrument Inc., Winooski, VT). For multilayers, cells were incubated with 40 μM BrdU for 48 hr. Thereafter, cells were fixed in 10% neutralized formalin and embedded in paraffin. The embedded tissues were cut into 5-μm sections, deparaffinized and immunohistochemically stained for BrdU incorporation, using a previously published method (19). The labeling index (LI) was determined microscopically and was defined as the number of BrdU-labeled nuclei divided by the total number of nuclei within a grid at 400X magnification. The LI was evaluated in the outermost 3–5 cell layers, where the cells were most actively labeled, to standardize the comparison. Note that the SRB assay is not suitable for measuring drug effect in multilayer cultures because it cannot be distinguish dead cells induced by drug treatment from the dead cells in the necrotic core (unrelated to drug treatment). Controls were processed similarly, with the exception of drug treatment.

Data Analysis

The concentration-effect relationship was analyzed using equation 1 (20).

\[ E = E_0 \left(1 - \frac{C}{K + C}\right) \]  

where \( E \) is the relative SRB reading in monolayers and LI in multilayers, \( C \) is the drug concentration, \( E_0 \) is the baseline effect in the absence of drug, \( K \) is the drug concentration at one-half \( E_0 \), and \( n \) is a curve shape parameter. Parameter values were estimated using nonlinear least squares regression (SAS Institute Inc., Cary, NC), and used to calculate the drug concentration needed to produce a 50% inhibition (IC\(_{50}\)).

Statistical Analysis

The differences between groups were determined by the Student's t-test. The one-sample t-test was used to determine if the ratios of IC\(_{50}\) between MMC and BMS were different from unity.

RESULTS

Kinetics of MMC and BMS Cytotoxicity in Monolayer Cultures

MMC and BMS produced sigmoidal concentration-dependent reduction of cell numbers in RT4 and TCC-SUP monolayers, immediately and up to 48 hr after the 2 hr drug treatment (Figure 1). The maximal effects of both drugs were approximately 100%. Table 1 summarizes the IC\(_{50}\) values. On a molar basis, BMS was 4 to 195 fold more cytotoxic than MMC in RT4 and TCC-SUP cells, at 0 to 48 hr post-treatment (p < 0.05 in all cases). The apparent activity of the two drugs increased with increased post-treatment time in both cell lines, indicating a delayed drug effect. MMC and BMS showed different extents and kinetics of the delayed effect, as follows. The ratios of IC\(_{50}\) for immediate-to-delayed effects were higher for MMC (up to 650 fold) than for BMS (up to 44 fold). The BMS activity was at or near its peak at 12 hr post-treatment, with minor changes at 24 and 48 hr (i.e. ≤50%). In contrast, the MMC activity continued to increase substantially with time; its effect in RT4 and TCC-SUP cells at 48 hr post-treatment was 12 and 60 times the effect at 12 hr.

Activity of MMC and BMS in Multilayer Cultures

MMC and BMS also produced sigmoidal and nearly complete concentration-dependent inhibition of BrdU incorporation in multilayers (Figure 2). As in monolayers, BMS showed greater activity than MMC against RT4 and TCC-SUP multilayers (Table 2). But the difference between BMS and MMC effects in multilayers was much smaller than in monolayers; BMS was only 2–3 times as active as MMC.

Chemosensitivity of Noninvasive and Invasive Tumor Cells

In nearly all experimental conditions (i.e. monolayer and multilayer cultures, drug effect measured at different post-treatment times), the invasive TCC-SUP cells were consistently and significantly less sensitive than the noninvasive RT4 cells to MMC and BMS (p < 0.05). The only exception was for the immediate effect of BMS in monolayers where the two cell lines were equally sensitive (p > 0.5).

DISCUSSION

Data of the present study show a higher potency of BMS, relative to MMC, against monolayer cultures of noninvasive and invasive bladder cancer cells. This is in agreement with the literature data obtained using monolayer cultures of other human cell lines (7–10). Our data further provide the new information that BMS was also more active than MMC in multilayer cultures which, because of their three-dimensional geometry, are structurally more similar to solid tumors than the two-dimensional monolayer cultures. The different potencies of MMC and BMS may be related to their different action mechanisms and activation pathways/kinetics (8,10,13). We have shown that presentation of inadequate MMC concentration