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-(nitrophenyldithio)-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 cytopathology 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 Biogenex (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% CO_2 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 10^7 cells were pipetted onto a piece of collagen gel (1 cm^2) 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

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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 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, VE). 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 section, 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).

$$E = E_0\left(1 - \frac{C^n}{K^n + C^n}\right) \quad (1)$$

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

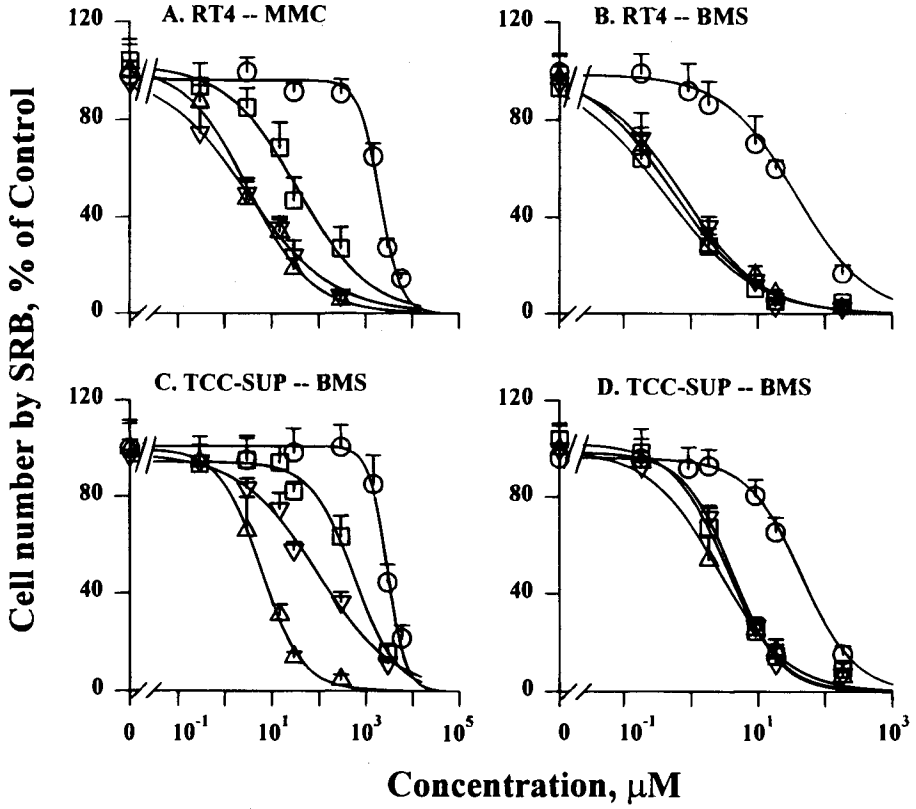

Fig. 1. Kinetics of drug-induced cytotoxicity. The concentration-effect relationships of MMC and BMS in RT4 cells (A and B) and TCC-SUP cells (C and D), measured by SRB assay immediately after 2 hr treatment (○), 12 hr (□), 24 hr (▽) and 48 hr (△) after treatment. Mean + SEM of a representative experiment (n = 3 for each data point).

to tumors located in bladder wall is a likely cause of treatment failure (1). The higher potency of BMS implies that less drug is required at the tumor site to produce a therapeutic effect, and represents a therapeutically exploitable advantage. Furthermore, the greater lipophilicity of BMS compared to MMC, as hinted by the lower solubility of BMS in aqueous solvents (data not shown), is likely to yield more favorable penetration across the urothelium and hence higher concentrations in bladder tissues. Additional studies are needed to determine if intravesical BMS therapy delivers adequate drug concentration to urothelium/lamina propria where superficial tumors reside and to muscle layers where invasive tumors reside.

Table 1. Different Kinetics of Cytotoxicity of MMC and BMS in RT4 and TCC-SUP Monolayers

Cell line	Post-treatment hr	IC_{50}[a], μM		Ratio of IC_{50}[b] MMC:BMS	Ratio of IC_{50}[c] immediate:delayed	
		MMC	BMS		MMC	BMS
RT4	0	1975 ± 166	23.4 ± 2.3	85.3 ± 16.1	NA	NA
	12	37.5 ± 4.8	0.6 ± 0.2	65.8 ± 28.4	53.7 ± 11.9	35.2 ± 17.4
	24	3.7 ± 0.5	0.9 ± 0.04	4.2 ± 0.5	545 ± 65.7	27.0 ± 2.1
	48	3.1 ± 0.6	0.6 ± 0.1	5.7 ± 1.6	656 ± 215	43.5 ± 10.5
TCC-SUP	0	2800 ± 168	21.2 ± 4.1	136 ± 30.7	NA	NA
	12	733 ± 64.7	3.8 ± 0.6	196 ± 31.1	3.8 ± 0.2	5.7 ± 1.7
	24	78.7 ± 9.8	3.3 ± 1.1	25.4 ± 8.2	35.9 ± 3.8	7.4 ± 4.5
	48	11.6 ± 5.4	2.5 ± 0.1	4.8 ± 2.3	285 ± 146	8.6 ± 1.4

Note: Cytotoxicity in monolayers was measured by the SRB assay, at 0 to 48 hr after the 2 hr drug treatment. A lower IC_{50} indicates an enhanced apparent activity. Mean ± SD of 3–6 experiments, each with triplicate samples. NA: not applicable. MW: 343 for MMC and 547.6 for BMS.

[a] $p < 0.05$ for differences between MMC and BMS effects at all post-treatment times.

[b] Different from 1 ($p < 0.05$) for all treatments.

[c] Ratios for MMC were higher than ratios for BMS at 24 and 48 hr post-treatment ($p < 0.05$), but not different at 12 hr post-treatment.

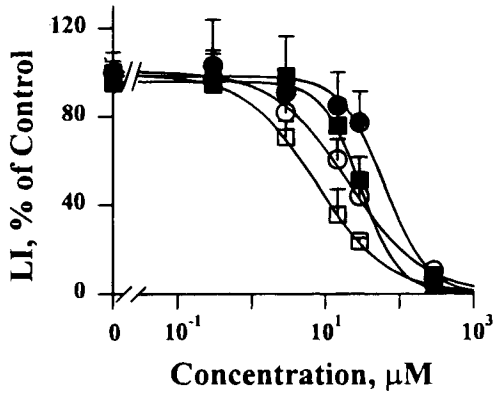

Fig. 2. Cytotoxicity of MMC (circles) and BMS (squares) in RT4 (open symbols) and TCC-SUP (closed symbols) multilayers. Drug treatment was for 2 hr, and drug effect was measured by inhibition of BrdU incorporation over 48 hr. Mean + SEM of a representative experiment (n = 3 for each data point).

The greatest difference in the activities of BMS and MMC was observed in monolayers when the drug effect was measured at 0 and 12 hr post-treatment, i.e. average of 75 fold in RT4 cells and 150 fold in TCC-SUP cells (Table 1). The differences became smaller for the drug effects in monolayers at 24 or 48 hr post-treatment and in multilayers where the cytotoxicity was measured over 48 hr; in which cases BMS was only 2 to 25 times as active as MMC (Tables 1 and 2). The time-dependent changes in the ratios of MMC:BMS effects are likely due to the different extents of delayed cytotoxicity under different experimental conditions (see next paragraph). Other possible causes for the different drug effects in monolayers and multilayers include biochemical differences due to the three-dimensional geometry and cell-to-cell interaction in multilayers. These include difference in drug and nutrient penetration and proliferative fraction (15,21,22).

The cytotoxicity of MMC and BMS detected immediately after treatment and the significant enhancement of apparent activity at 12 to 48 hr post-treatment indicate that these drugs caused immediate and delayed cytotoxicity. The substantially greater reduction in the IC_{50} of MMC with time compared to BMS indicates that the contribution of the delayed cytotoxicity was more predominant for MMC than for BMS. There are a number of possible mechanisms that may account for the different cytotoxicity kinetics of the two drugs, including a different rate of activation with the non-enzymatic thiol reduction of BMS being more rapid than the quinone reductase-mediated activation of MMC, different rates of producing DNA breaks and cross-links by the activated species, and/or different rates of repairing the damaged DNA.

Table 2. Comparison of MMC and BMS Activities in RT4 and TCC-SUP Cell Multilayers

Cell line[a]	IC_{50}[b], μM		Ratio of IC_{50}[c]
	MMC	BMS	MMC:BMS
RT4	24.9 ± 1.4	7.6 ± 1.6	3.4 ± 0.6
TCC-SUP	59.3 ± 1.0	28.3 ± 6.7	2.2 ± 0.6

Note: Cells were treated for 2 hr with drugs. Cytotoxicity in multilayers was measured as the inhibition of BrdU incorporation over 48 hr. Mean ± SD of 3–6 experiments, each with triplicate samples.

[a] $p < 0.05$ for differences between the response of RT4 and TCC-SUP cells to either MMC or BMS.

[b] $p < 0.05$ for differences between MMC and BMS effects, for either RT4 or TCC-SUP cells.

[c] Different from 1 ($p < 0.05$) for MMC and BMS.

In general, monolayer and multilayer cultures of the invasive TCC-SUP cells were less sensitive than non-invasive RT4 cells to MMC and BMS (Tables 1 and 2). The data on MMC is consistent with our reported lower sensitivity of invasive human bladder tumors compared to superficial tumors (2). While the causes for differential sensitivity to BMS are not known, the differential sensitivity to MMC may be due to deficient drug activation and/or increased repair of DNA damage in TCC-SUP cells relative to RT4 cells. For example, the activities of NADPH cytochrome p450 reductase and DT diaphorase and the frequency of interstrand DNA cross-link in invasive human bladder tumor cells were only 8%, 31% and 60%, respectively, of those in noninvasive tumor cells (8).

In conclusion, the higher cytotoxicity of BMS than MMC in both 2- and 3-dimensional cultures as well as the more rapid induction of maximal cytotoxicity indicate different activity profiles for the two drugs. To explore the potential benefits of these differences, additional studies to further develop BMS as an alternative to MMC for use in intravesical bladder cancer therapy are warranted.

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REFERENCES

1. M. G. Wientjes, J. T. Dalton, R. A. Badalament, J. R. Drago, and J. L.-S. Au. *Cancer Res.* **51**:4347–4354 (1991).
2. T. D. Schmittgen, J. M. Weaver, R. A. Badalament, R. A., M. G. Wientjes, E. Klein, D. Young, D., and J. L.-S. Au. *J. Urol.* **152**:1632–1636 (1994).
3. M. G. Wientjes, R. A. Badalament, and J. L.-S. Au. *Cancer Chemother. Pharmacol.* **32**:255–262 (1993).
4. S. R. Keyes, P. M. Fracasso, D. C. Heimbrook, S. Rockwell, S. G. Sligar, and A. C. Sartorelli. *Cancer Res.* **44**:5638–5643 (1984).
5. R. R. Perry, Y. Kang, and B. Greaves. *Biochem. Pharmacol.* **46**:1999–2005 (1993).
6. S. Pan, S. A. Akman, G. L. Forrest, C. Hipsher, and R. Johnson. *Cancer Chemother. Pharmacol.* **31**:23–31 (1992).
7. B. H. Xu, V. Gupta, and S. V. Singh. *Int. J. Cancer.* **58**:686–692 (1994).
8. B. H. Xu, V. Gupta, and S. V. Singh. *Br. J. Cancer.* **69**:242–246 (1994).
9. L. Dusre, S. Rajagopalan, H. M. Eliot, J. M. Covey, and B. K. Sinha. *Cancer Res.* **50**:648–652 (1990).
10. B. H. Xu and S. V. Singh. *Cancer Res.* **52**:6666–6670 (1992).
11. W. T. Bradner, W. C. Rose, J. E. Schurig, and A. P. Florczyk. *Invest. New Drugs.* **8**:S1–S7 (1990).
12. Q.-Y. He, H. Maruenda, and M. Tomasz. *J. Am. Chem. Soc.* **116**:9349–9350 (1994).
13. S. Rockwell, B. Kemple, and M. Kelley. *Biochem. Pharmacol.* **50**:1239–1243 (1995).

14. C. L. Bregman, R. A. Buroker, W. T. Bradner, R. S. Hirth, and H. Madisson. *Fundament. Appl. Toxicol.* **13**:46–64 (1989).
15. W. C. Yen, T. Schmittgen, and J. L.-S. Au. *Pharm. Res.* In press (1996).
16. R. Hay, J. Caputo, T. R., Chen, M. Macy, P. Mclintock, and Y. Reid. American-Type Culture Collection. 7th ed., 1992.
17. C. O'Toole. In: Webber, M. M. (ed), *In vitro models for cancer research*, CRC Press, Vol. IV, 1986, p. 103–125.
18. P. Skehan, R. Storeng, S. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, and M. R. Boyd. *J. Natl. Cancer Inst.* **82**:1107–1112 (1990).
19. Y. Gan, M. G. Wientjes, D. E. Schuller, and J. L.-S. Au. *Cancer Res.* **56**:2086–2093 (1996).
20. N. H. Holford and L. B. Sheiner. *Pharmacol. Ther.* **16**:143–166 (1982).
21. R. M. Hoffman. *Cancer Metast. Rev.* **13**:169–173 (1994).
22. P. S. Steeg, M. C. Ally, and M. R. Grever. *J. Natl. Cancer Inst.* **86**:953–955 (1994).