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CYTOTOXICITY OF BMS-181174

EFFECTS OF HYPOXIA, DICOUMAROL, AND REPAIR DEFICITS

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Abstract—The mitomycin C (MC) analog BMS-181174 (previously designated as BMY25067) has been shown to be active against a variety of solid tumors in mice. The activity of this compound against tumor cell lines resistant to MC and the different toxicity profiles of BMS-181174 and MC suggested that there may be significant differences in the metabolism and the mechanisms of action of these two compounds. Our studies with a mouse mammary tumor cell line (EMT6), a wild-type Chinese hamster cell line (AA8), and three repair-deficient Chinese hamster cell lines (UV4, UV5, and EM9) supported this concept. BMS-181174 was more toxic to all five cell lines in air than in hypoxia; in contrast, MC is more toxic in hypoxia. Dicoumarol (which increases the cytotoxicity of MC in hypoxia and reduces the cytotoxicity of this drug in air) did not alter the cytotoxicity of BMS-181174. This finding suggests that neither DT-diaphorase nor cytochrome b_5 reductase is involved in the activation of BMS-181174. Studies with the repair-deficient cell lines suggest that DNA strand breaks are not important to the cytotoxicity of BMS-181174, and that cross-links and adducts may be the critical lesions; these studies also suggest that the lethal lesions produced by BMS-181174 are the same under aerobic and hypoxic conditions.

Key words: BMS-181174; BMY25067; mitomycin analogs; bioreductive alkylating agent; quinone reductase

N-7[2-(nitrophenyldithio)-ethyl]mitomycin C (BMS-181174; previously BMY25067) is currently in clinical trials as an antineoplastic agent [1, 2]. Like MC,† this agent shows broad-spectrum activity against a variety of transplanted tumors in mice [3–5]. However, BMS-181174 can be activated by homogenates of MC-resistant cells and is active against several rodent and human cell lines that have intrinsic or acquired resistance to MC [3–6]. Preclinical toxicology studies also reveal very significant differences between BMS-181174 and MC [3, 7, 8]. The maximally tolerated dose of BMS-181174 in rodents is 1.5 to 2 times that of MC. The dose-limiting toxicity of BMS-181174 in rodents is myelosuppression; little cardiac, renal, and pulmonary toxicity has been observed. These data suggest that the metabolism and mechanism of action of BMS-181174 may differ from those of MC.

Biochemical studies point to the same conclusion [8–11]. Studies with extremely high concentrations of BMS-181174 in isolated hepatocytes have been interpreted as suggesting that the cytotoxicity of BMS-181174, as assessed by trypan blue exclusion, may be attributed to oxidative stress leading to mitochondial toxicity [10]. Studies with cardiac microsomes and perfused hearts [8] and experiments with drug-resistant MCF7 cells [6] may likewise suggest a role for free-radical damage. However, data for MCF7 cells also suggest that DNA alkylation may be a critical cytotoxic lesion [6]. Recently, Tomasz and her collaborators [11] reported a non-enzymatic, thiol-initiated pathway of reduction that can activate BMS-181174 to a species capable of forming cross-links in DNA. These conflicting data on the effects of BMS-181174 led us to perform the studies reported here, in an effort to elucidate the pathways of activation and the mechanism of cytotoxicity of BMS-181174 in living cells.

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† Abbreviations: MC, mitomycin C; POR, porfiromycin; and DIC, dicoumarol.

MATERIALS AND METHODS

Cells

EMT6 mouse mammary tumor cells (subline EMT6 Rw) were maintained as monolayers in Waymouth's medium (Gibco, Grand Island, NY) supplemented with 15% fetal bovine serum (Hyclone, Logan, UT) and antibiotics, in a 37°, humidified, 95% air/5% CO_2 atmosphere. The techniques used to handle and maintain these cells are described in detail elsewhere [12–13].

The effects of BMS-181174 were also examined using a wild-type Chinese hamster cell line, AA8, and three sublines with defects in specific DNA repair systems: EM9, which is deficient in the repair of single-strand breaks; UV4, which has a very low efficiency in removing cross-links; and UV5, which has a milder deficit in cross-link repair and a defect in the repair of bulky monoadducts [14–18]. These cell lines were a gift from Dr. Larry Thompson at Lawrence Livermore National Laboratory and were propagated as monolayers in α-minimum essential medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone) as described previously [14–18]. These cells have been used in our past studies examining the effects of MC, POR, and decarbamoyl mitomycin C [17, 18].

Monolayer cultures for experiments were initiated by plating 2×10^5 cells into glass milk dilution bottles and were used 3–4 days later, in exponential growth. On the day of drug treatment, the medium was replaced with

fresh medium, and the flasks were divided into two groups: one treated in an aerobic environment and the other treated under hypoxic conditions [13, 19–21]. Cultures to be exposed in hypoxia were stoppered with tight-fitting rubber sleeves and were gassed with a humidified mixture of 95% N_2/5% CO_2 (<10 ppm O_2). Previous studies have shown that this method produces severe hypoxia after 1 hr. Cultures for these experiments were gassed with N_2/CO_2 for 2 hr, and then treated with graded doses of drug for 1 hr. Drugs were injected through the septums of the rubber stoppers without interrupting the flow of N_2/CO_2; this procedure did not introduce sufficient oxygen to alter the radiosensitivity of the hypoxic cells and did not alter the pH of the culture medium. Aerobic cultures were treated analogously, but in 95% air/5% CO_2.

At the end of the drug treatment, the medium was removed, and the cells were washed, trypsinized, and suspended. The single cell suspension was then counted using a Coulter Counter model ZBI. One to four dilutions were made and plated, in quadruplicate, in 60 mm tissue culture dishes (Costar). Cultures were incubated for 1 or 2 weeks for Chinese hamster cells or EMT6 cells, respectively, to allow viable cells to grow in macroscopic colonies [12, 17]. The medium was then removed, the cultures were washed with 0.9% saline, and the colonies were fixed and stained with a solution of crystal violet, formalin, and methanol. Colonies containing more than 40 cells were counted. Surviving fractions for treated cultures were calculated using the cloning efficiencies of untreated controls assayed on the same day [12]. Cloning efficiencies in these experiments averaged 78 ± 5% for EMT6 cells, 68 ± 5% for AA8 cells, 81 ± 2% for EM9 cells, 84 ± 8% for UV4 cells and 93 ± 7% for UV5 cells. Cell numbers in treated and control cultures were compared to test for rapid cytolysis in drug-treated cultures, during treatment or trypsinization, which might complicate analyses of clonogenicity [12]; none was observed in these experiments. Vehicle-treated controls, subjected to all of the experimental conditions and treated with maximal concentrations of vehicles, were also included in each experiment. Under aerobic conditions, the surviving fractions for the vehicle-treated controls were not significantly different from 1; the surviving fractions for controls treated with vehicles plus hypoxia were slightly lower, as shown on the figures.

Drugs

BMS-181174 (BMY25067), N-7[2-(4-nitrophenyldithio)-ethyl]mitomycin C, was a gift from the Bristol-Myers Squibb Co. (Wallingford, CT). The structure of BMS-181174 is shown in Fig. 1. BMS-181174 was dissolved in DMSO and then diluted in medium. The stock was kept frozen, protected from light, and used within 2 weeks. DIC, purchased from the Sigma Chemical Co. (St. Louis, MO), was dissolved in 0.5 N NaOH. Stock solutions were stored frozen and used within 2 months.

RESULTS

The cytotoxicity of BMS-181174 to EMT6 cells was significantly greater under aerobic conditions than under acute hypoxia (Fig. 2). This selective aerobic cytotoxicity contrasts with the effects of MC and POR, both of which are more toxic to hypoxic cells than to aerobic

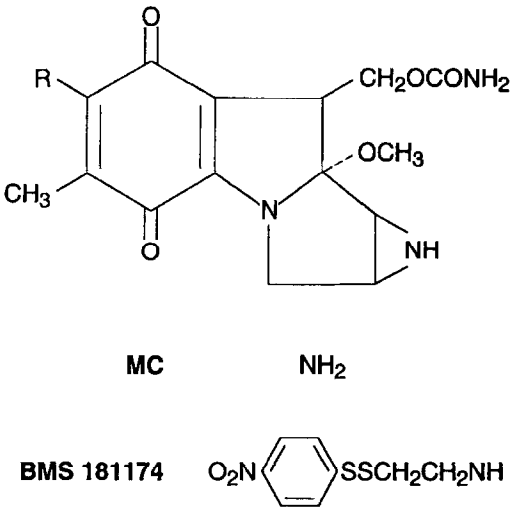

Fig. 1. Structures of MC and BMS-181174.

cells [17–24]. In aerobic cells, BMS-181174 was more toxic than MC: a 1-hr treatment with 3 μM MC produced a surviving fraction of ~0.03 [21], about 100-fold higher than the survival obtained with the same concentration of BMS-181174 in the studies shown in Fig. 2. In contrast, BMS-181174 in hypoxia was considerably less toxic than MC in hypoxia: a 1-hr treatment with 3 μM MC produced a surviving fraction of ~0.002, [21], ~100-fold lower than the survival obtained with the same concentration of BMS-181174 in the studies shown in Fig. 2. The reversed hypoxic/aerobic differential seen with BMS-181174 therefore reflects both a greater cytotox-

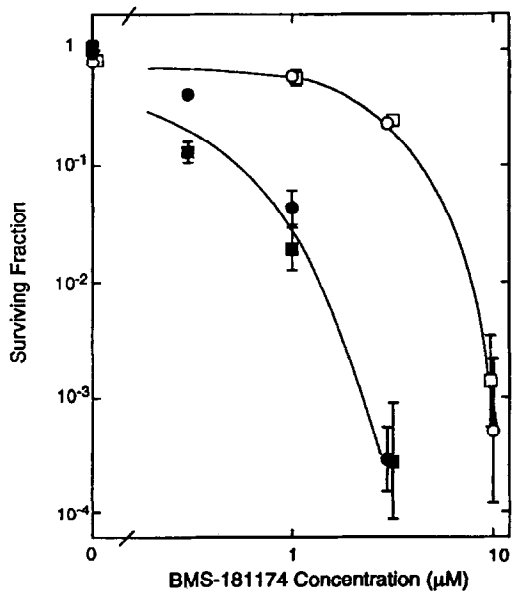

Fig. 2. Survival of EMT6 cells treated for 1 hr with BMS-181174 under aerobic conditions (●, ■) or in severe hypoxia (○, □). The survivals of cells treated with BMS-181174 alone (●, ○) and those of cells also treated simultaneously with 300 μM dicoumarol and BMS-181174 (■, □) were indistinguishable. Points are means of 3 or more independent experiments. SEMs are shown where larger than points.

icity of this compound in air and a lesser cytotoxicity in hypoxia.

Figure 2 also shows survival curves for EMT6 cells treated with BMS-181174 in the presence of 300 μM DIC. At this concentration, DIC is a potent inhibitor of both DT-diaphorase [NAD(P)H:quinone oxidoreductase; EC 1.6.99.2] and NADH:cytochrome b_5 oxidoreductase [EC 1.6.2.2], two enzymes known to be capable of activating MC [25–28], and has a very significant effect on the cytotoxicity of MC and POR, increasing the cytotoxicities of these drugs in hypoxia and decreasing their cytotoxicities in air [25, 29–31]. In contrast, simultaneous treatment with 300 μM DIC had no significant effect on the cytotoxicity of BMS-181174 either in air or in hypoxia: the survival curves for BMS-181174 alone were indistinguishable from those obtained in the same experiments when 300 μM DIC was added immediately before the addition of BMS-181174. This finding implies that there are very significant differences in the pathways of activation of BMS-181174 and MC by EMT6 cells both in air and in hypoxia.

To ascertain whether the preferential toxicity of BMS-181174 to aerobic cells was cell line specific, the differential toxicity of this drug was also examined using AA8 cells, a wild-type Chinese hamster cell line used by ourselves [17, 18] and others [14, 32] to study bioreductive drugs. BMS-181174 was more toxic to AA8 cells in air than in hypoxia (Fig. 3). Aerobic AA8 cells were markedly more sensitive to BMS-181174 in these studies than they were to MC in our previous studies [17, 18]. The sensitivities of hypoxic AA8 cells to the two agents did not differ dramatically.

The pattern of the cytotoxicity of BMS-181174 was examined in three cell lines derived from AA8, which have specific defects in the repair of damaged DNA. In the EM9 cells, which are defective in the repair of single- and double-strand breaks, BMS-181174 was again more effective in air than under hypoxic conditions (Fig. 3). There were no significant differences between the survival curves for EM9 and AA8 cells treated with BMS-181174. The similarities of the potencies and aerobic/hypoxic differentials of BMS-181174 in the wild-type cell line and in this strand-break-repair deficient cell line suggest that DNA strand breaks are not major cytotoxic lesions after treatment with this compound under either aerobic or hypoxic conditions.

The UV5 cell line, which is defective in its ability to repair monoadducts, was somewhat more sensitive than AA8 cells to BMS-181174 under both aerobic and hypoxic conditions (Fig. 3). The UV4 cell line, which has a much more severe defect in cross-link and adduct repair, was hypersensitive to BMS-181174 (note the 10-fold change in concentrations on the X-axis of this panel in Fig. 3). In both of these cell lines, the cytotoxicity of BMS-181174 was greater in air than in hypoxia. The sensitivity of these cell lines to BMS-181174 suggests a major role for cross-links and a possible role for monoadducts in the cytotoxicity of this compound.

DISCUSSION

The data presented in this report confirm the findings of others, which suggest major differences between the metabolism and effects of BMS-181174 and those of MC [1–11]. The preferential toxicity of BMS-181174 to aerobic cells is in marked contrast to the hypoxia-selective toxicity observed for MC and POR in these cell lines [13, 17–19, 25, 29–32]. The preferential toxicity of BMS-181174 in air was not cell line specific; rather, similar differential toxicities were observed in all five cell lines examined in these studies. Selective toxicity of BMS-181174 to aerobic EMT6 cells has also been observed by Sartorelli* and by ourselves in independent experiments.† Interestingly, the closely related MC analog BMY25282 [7-N-(dimethylaminomethylene)-mitomycin C] also has preferential toxicity to aerobic EMT6 cells [20, 21]. BMY25282 is more toxic to aerobic EMT6 cells than is either MC or BMS-181174, but is not dissimilar to MC in its toxicity to hypoxic cells. Thus, in EMT6 cells as in other systems [4–8], there appear to be

* Personal communication from A. C. Sartorelli. Cited with permission.

† Rockwell S and Kelley M, Manuscript submitted for publication.

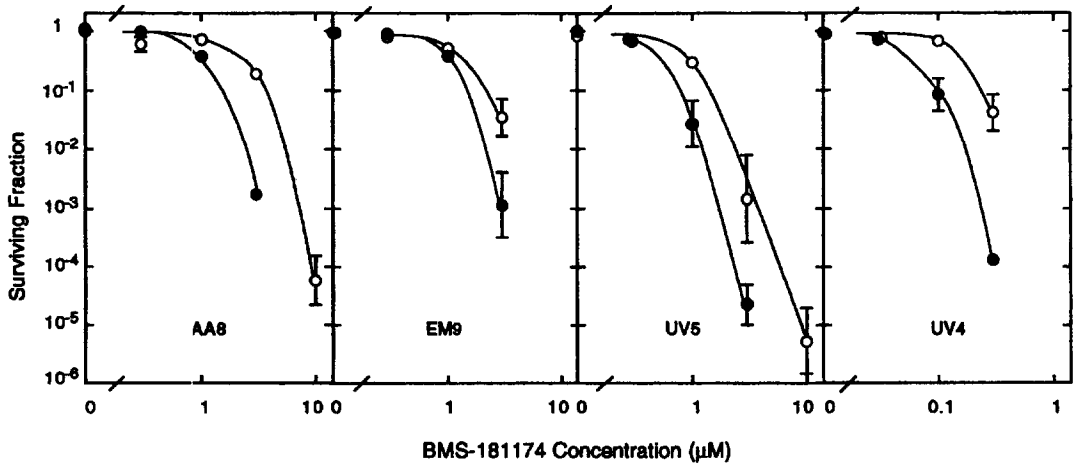

Fig. 3. Survival of AA8, EM9, UV5, and UV4 cells treated with BMS-181174 in air (●) and hypoxia (○). Points are means of 3 or more independent experiments. SEMs are shown where larger than points. Note that the concentrations shown on the X-axis of the panel showing data for UV4 cells differ from those used in the panels for the other cell lines.

differences between the effects of these two closely related N-7 amino derivatives of MC.

Dicoumarol had no discernible effect on the cytotoxicity of BMS-181174 in either air or hypoxia. Again, this observation provides evidence that the metabolism of this compound differs dramatically from the metabolism of MC and POR. The sensitivity of EMT6 cells to both of these drugs is increased by DIC in hypoxia and is decreased by DIC in air [25, 29, 30, 33]. Because DIC at the concentration used in these experiments inhibits both cytochrome b_5 reductase and DT-diaphorase [25–28], these data suggest that neither of these enzymes plays a critical role in the metabolism of BMS-181174 in EMT6 cells under either aerobic or hypoxic conditions.

The survival curves for the Chinese hamster cell lines provide some insights into the cytotoxic lesions produced by this drug. The similarity of the sensitivities of EM9 and AA8 cells argues against a major role for free radical damage to DNA as a mechanism of cytotoxicity, because such oxidative radical damage generally produces large numbers of single-strand and double-strand DNA breaks, which contribute to cytotoxicity. EM9 cells are deficient in the repair of strand breaks and, therefore, are sensitive to agents, such as radiation, that produce these lesions [14–16]. If the greater aerobic toxicity of BMS-181174 were to reflect increased redox cycling and, therefore, greater radical-induced strand breakage in DNA, one would expect to see a greater potency of BMS-181174 in aerobic EM9 cells than in aerobic AA8 cells. Moreover, if redox cycling producing DNA strand breaks occurred only under well-oxygenated conditions (as might be predicted from most O_2-dependent models of redox cycling), one would also predict a larger differential between the aerobic and hypoxic cytotoxicities of BMS-181174 in EM9 cells than in AA8 cells; this was not observed.

The cell lines deficient in the repair of monoadducts and cross-links were more sensitive to BMS-181174 than were the wild-type cells. The severely cross-link repair-deficient UV4 cell line was exquisitely sensitive to BMS-181174, as it was to MC, POR, and decarbamoyl MC [17, 18]. It is interesting, however, that for BMS-181174 the aerobic/hypoxic differentials in the UV4 and UV5 cell lines were similar to that for AA8 cells. This finding stands in contrast to findings in similar studies with MC and POR [17]; the differential toxicities for MC and POR in the repair-deficient cell lines were significantly smaller than the differential for AA8 cells [17], in agreement with biochemical studies that suggested a change in the nature of the lesions produced by MC and POR in air and hypoxia [31]. The similarity of the aerobic/hypoxic differentials for BMS-181174 in the cell lines deficient in the repair of adducts and in the wild-type AA8 lines suggests that the critical cytotoxic lesions produced by this compound in air and hypoxia are qualitatively similar, and are produced in greater numbers under aerobic conditions.

The results of our studies are in accord with the conclusions drawn in a recent paper by Tomasz and her collaborators [11], who showed that BMS-181174 and a related analog, also carrying a disulfide on its quinone side chain, could be reduced in a chemical system, through a novel, non-enzymatic pathway of activation that was initiated by thiols, including glutathione. This thiol-induced activation occurred by a conceptually novel thiol-to-quinone electron transfer mechanism, and resulted in the rapid production of a species that cross-linked DNA. Our data are consistent with the hypothesis that analogous non-enzymatic thiol-initiated activation of BMS-181174 occurs in living cells, producing a similar bifunctional alkylating species, which produces a cytotoxic effect by cross-linking DNA. If this pathway were sufficiently rapid, the contribution to cytotoxicity of enzymatic activation via DT-diaphorase or cytochrome b_5 reductase could be insignificant, explaining our results with DIC. Similarly, the effectiveness of this thiol-initiated activation pathway in producing highly cytotoxic cross-links could be responsible for the notable potency of BMS-181174, which limited the drug concentrations usable in our clonogenicity studies to levels very much lower than those found by others [10] to produce oxidative stress. A simple predominant chemical pathway of activation could also result in a simpler spectrum of cytotoxic lesions for BMS-181174 than is found for MC, POR or other mitomycins that are reduced by several different enzymes, resulting in a variety of activated intermediates and potentially cytotoxic species. It is still unclear why BMS-181174 should be more toxic under aerobic conditions than in hypoxia. Further studies with intact cells and with chemical systems will be needed to examine this question.

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