Porfiromycin as a Bioreductive Alkylating Agent with Selective Toxicity to Hypoxic EMT6 Tumor Cells in Vivo and in Vitro

Susan R. Keyes, Sara Rockwell, and Alan C. Sartorelli

School of Medicine, New Haven, Connecticut 06510

ABSTRACT

Hypoxic cells may limit the curability of solid tumors by conventional chemotherapeutic agents and radiotherapy. Agents which are preferentially toxic to cells with low oxygen contents could therefore be useful as adjuncts to the regimens now used to treat these cancers. To date, the best agent of this type that we have tested is porfiromycin. Porfiromycin is similar to mitomycin C in its toxicity to hypoxic EMT6 cells in vitro but has much less toxicity than mitomycin C to well-oxygenated EMT6 cells. EMT6 cell sonicates reduce mitomycin C and porfiromycin to reactive electrophiles at similar rates under hypoxic conditions, a finding that correlates with cytotoxicity, whereas the rate of production of reactive species from both drugs is very slow under aerobic conditions. We also show that porfiromycin is capable of killing hypoxic radiation-resistant cells in solid EMT6 tumors. Appropriate regimens combining porfiromycin (which preferentially kills hypoxic cells) and radiation (which preferentially kills aerated cells) may therefore be especially efficacious for the treatment of solid tumors.

INTRODUCTION

Solid tumors in animals and humans contain hypoxic cells, which are resistant to radiation and to many chemotherapeutic agents and which limit the cure of these cancers by conventional therapeutic regimens (1, 2). To develop therapeutic approaches for attacking these oxygen-deficient cells, we have sought drugs which are reductively activated in the absence of oxygen to yield cytotoxic alkylating agents. We have shown previously that MC is preferentially toxic to hypoxic EMT6 carcinoma cells, Sarcoma 180 cells, and V79 Chinese hamster lung fibroblasts in vitro (3-6). Studies of the mechanism of bioactivation have demonstrated that enzymatic reduction of MC under hypoxic conditions generates a reactive moiety, which can be trapped by 4-(p-nitrobenzyl)pyridine (3, 4, 6); this alkylating species may produce cytotoxic lesions in hypoxic cells. Measurements of MC cytotoxicity in solid intradermal EMT6 tumors have shown that this antibiotic reaches and kills hypoxic tumor cells spared by radiotherapy (7, 8). These results suggest that measurement of the relative cytotoxicity of potential chemotherapeutic agents to aerobic and hypoxic cells in vitro may be relevant for identifying drugs which would be particularly effective against hypoxic tumor cells in vivo and therefore especially useful as adjuncts to radiotherapy.

Extending these studies to other quinone antibiotics has shown that porfiromycin, like MC, is preferentially toxic to hypoxic cells in vitro (9). Further testing of porfiromycin both in vivo and in vitro indicates that this agent is more selective than MC in its differential effects on hypoxic and aerobic cells in vitro and may produce a better therapeutic ratio when used as an adjuvant to radiotherapy in vivo. This paper presents the results of these studies.

MATERIALS AND METHODS

Porfiromycin and MC were gifts from Dr. Terry Doyle of Bristol Laboratories (SYN). All other materials were purchased from commercial sources: gases (Presto Welding Service Center, North Haven, CT); fetal bovine serum and Waymouth's medium (Grand Island Biological Co., Grand Island, NY); NADP+, glucose-6-phosphate dehydrogenase (type XII), and glucose-6-phosphate (Sigma Chemical Co., St. Louis, MO); and 4-(p-nitrobenzyl)pyridine (Aldrich Chemical Co., Milwaukee, WI).

Reactive metabolites of MC and porfiromycin were measured using sonicated EMT6 cells, by trapping the activated species with the nucleophile 4-(p-nitrobenzyl)pyridine, in a modification (3, 4) of the method of Wheeler et al. (10). The absorbance of the mitomycin C or porfiromycin 4-(p-nitrobenzyl)pyridine adduct(s) was measured at 540 nm.

All experiments were performed using EMT6 mouse mammary tumor cells, which were maintained by alternate passage in vivo and in vitro as detailed elsewhere (11). Cell culture studies were performed, using exponentially growing monolayers in glass milk dilution bottles, in Waymouth's medium supplemented with 15% fetal bovine serum and antibiotics. Hypoxia was produced by gassing the culture with a humidified mixture of 95% N2 and 5% CO2 for 2 h (3, 4, 6, 12). MC and porfiromycin were dissolved in 70% ethanol, and small volumes of drug were added to the hypoxic cultures without compromising the hypoxia as described previously (6). The viability of the cells was assayed by measuring the ability of individual cells to form colonies (11). Each experiment included controls subjected to all experimental manipulations and incubated with the vehicle used to dissolve the drug under appropriate aerobic and hypoxic conditions.

In vivo studies were performed using EMT6 tumors in 2.5- to 3-month-old BALB/c KaRw mice by previously published procedures (7, 11-13). Tumors were implanted by the injection of 2 x 106 cells into the skin of the flank and were allowed to grow for 2 weeks, producing tumors approximately 100 cu mm in volume. Porfiromycin was administered i.p. at doses of 3, 6, 12, 20, 30, or 40 µg/g, 1 h before sacrifice. Mice were whole body irradiated, without anesthesia, with 15 Gy of 250 keV X-rays at a dose rate of approximately 2 Gy/min (7, 12, 14). Radiation was begun 50 min after the drug treatment. Previous studies demonstrated that the toxicities of MC and radiation in vitro were independent and did not depend on the sequence or timing of the MC and radiation treatments (15) and that intervals of 5 min to 24 h between MC and X-rays in vivo gave similar cytotoxicities (7). Tumors were excised immediately after irradiation or 1 h after porfiromycin treatment. Tumor cells were suspended by a combination of mechanical and enzymatic disruption and were collected by centrifugation, counted, and assayed for viability by determining their ability to form colonies in vitro (7, 11, 12).
RESULTS

The cytotoxicities of porfiromycin and mitomycin C to EMT6 cells were measured under both aerobic and hypoxic conditions (Chart 1). At concentrations as low as 0.5 μM, a 1-h treatment with porfiromycin was significantly more toxic to cells at low oxygen concentrations than to well-oxygenated cells. This difference in cell viability increased dramatically as the porfiromycin concentration increased. The cell survival curve for treatment with porfiromycin under hypoxic conditions declined steeply, while the curve defining the aerobic cytotoxicity of porfiromycin was shallow and possibly complex. Chart 1 also includes data defining the cytotoxicity of MC, which were obtained in the experiments used to study porfiromycin and are similar to more extensive data published previously (5, 6). Under hypoxic conditions, the survival curves for MC and porfiromycin were virtually identical. In contrast, MC was significantly more toxic than porfiromycin to aerobic cells. Similar survival curves were obtained when aerobic and hypoxic EMT6 cells were exposed to 1 μM porfiromycin for various lengths of time (Chart 2). In these studies, porfiromycin showed a selective toxicity to hypoxic cells with as little as 0.5 h of exposure to drug, with the difference in the cell viabilities increasing with increasing durations of drug exposure.

DNA cross-links have been hypothesized to be the lesions responsible for the cytotoxicity of the mitomycins (16–20); because of this, we measured the rate of generation of reactive metabolites from MC and porfiromycin using EMT6 cell sonicates by monitoring the rate of alkylation of 4-(p-nitrobenzyl)pyridine (Chart 3). Using MC, this assay was shown previously to respond linearly with changes in protein concentration or drug concentration (4). The rate of production of reactive electrophiles from porfiromycin was linear for up to 30 min in hypoxia. Similar results obtained with MC agree with data published previously.

![Chart 1](image1.png)

![Chart 2](image2.png)

![Chart 3](image3.png)
PORFIROMYCIN TOXICITY TO HYPOXIC TUMOR CELLS

Chart 4. Survival of cells from EMT6 tumors in mice treated with porfiromycin (C) or porfiromycin and X-irradiation (D). Points, mean of 3 or more independent determinations; bars, SE. Surviving fractions were calculated using the plating efficiencies of cells from untreated control tumors, which averaged 45 ± 1%.

(3, 4, 6). The rates of production of alkylating species from MC and porfiromycin at equimolar concentrations (300 μM) were 1.37 and 1.40 A₄₅₀ 10⁻³ min⁻¹ mg⁻¹, respectively. Under aerated conditions, the rates of production of alkylating species from MC and porfiromycin were much lower, 0.054 and 0.047 A₄₅₀ 10⁻³ min⁻¹ mg⁻¹, respectively.

The response of solid EMT6 tumors in vivo to porfiromycin is shown in Chart 4. The survival curve for tumor cells explanted from mice treated with porfiromycin alone had a very shallow slope. This reflects the fact that approximately 80% of the viable cells in EMT6 tumors are aerobic (2); the overall response of these tumors to porfiromycin is therefore dominated by the response of the aerobic tumor cells. Aerobic tumor cells in vivo, like those in vitro (Charts 1 and 2), were relatively resistant to porfiromycin. To examine the effect of porfiromycin on hypoxic tumor cells in vivo, we selectively depleted the aerobic tumor cell population by treating the tumors with a large dose of X-rays (15 Gy); this reduced the survival of the tumor cells to 3.8% (Chart 4). Because hypoxic cells are exceedingly resistant to radiation, while aerobic cells are radiosensitive (2, 12, 14), this treatment reduces the survival of the aerated tumor cell population to approximately 0.01%, while approximately 20% of the hypoxic tumor cells survive (2, 12). Therefore, the tumor cell population surviving irradiation is composed almost entirely of hypoxic cells. The effect of porfiromycin on the hypoxic (radioresistant) tumor cells was greater than the effect on the overall tumor cell population.

Isobologram analyses, performed using complete dose-response curves for porfiromycin alone (Chart 4) and for radiation alone (2, 8, 11, 14), indicated that both the combination of 6 μg of porfiromycin per g plus 15 Gy of X-rays and the combination of 20 μg of porfiromycin per g plus 15 Gy of X-rays produced responses which lay just outside the area of additivity and were compatible with a supraadditive effect (21). This probably reflects the fact that each agent is effective against a tumor cell population which is resistant to the other agent.

A similar conclusion could be drawn from the data presented in Chart 4. If, for example, one calculates the predicted survival for the combination of 20 μg of porfiromycin per g plus 15 Gy of X-rays as the product of the survivals for each agent, one would predict that the combination would give a surviving fraction of approximately 0.0035; the survival actually observed with the combination is far lower. This analysis is less rigorous than the isobologram analysis described above, because it fails to consider the shapes of the 2 dose-response curves but only considers survivals at the individual doses studied; this can lead to erroneous estimations if either dose-response curve is nonlinear (21). In this case, however, both methods of analysis indicate that the response of the tumor to the combination of porfiromycin and X-rays is greater than would be expected on the basis of additive cytotoxicities.

DISCUSSION

The structure of porfiromycin differs from that of MC by the simple addition of a methyl group on the aziridine ring nitrogen; the 2 compounds have virtually identical oxidation-reduction potentials of −0.40 V. We therefore expected them to act in a similar manner. Under hypoxic conditions, MC and porfiromycin were essentially equitoxic to EMT6 cells in vitro. The equivalent cytotoxicities of these drugs to cultured cells in hypoxia were paralleled by similar rates of production of reactive electrophiles from the drugs by hypoxic cell sonicates. The reactive electrophiles presumably are capable of alkylating DNA and may be responsible for the cytotoxicity of these quinones in hypoxic cells. The cytotoxicities of MC and porfiromycin differed markedly in well-oxygenated EMT6 cells. The lesser cytotoxicity of porfiromycin in the presence of oxygen resulted in an overall increase in the selectivity of porfiromycin for hypoxic cells. As a result, porfiromycin should be superior to MC as a therapeutic agent for selectively attacking hypoxic tumor cells.

This hypothesis was tested using solid EMT6 tumors; the viable (clonogenic) cell population in these tumors includes both radiation-resistant, hypoxic cells (20%) and radiation-sensitive, well-oxygenated cells (80%) (2, 12). The overall response of the tumors to porfiromycin used as a single agent was smaller than the response to MC reported previously (7). This finding agrees with the relative toxicity of the 2 drugs to aerobic cells in culture and with the relative toxicity of the 2 drugs to mice (22). We examined the effect of porfiromycin on the hypoxic tumor cells by using 15 Gy of radiation to deplete the aerobic tumor cells, leaving a surviving cell population composed almost entirely of hypoxic cells (2, 14). We observed that a low dose of porfiromycin (6 μg/g) had an effect on the hypoxic cells similar to that reported previously (7) for the same dose of MC. However, the LD₅₀ for MC in mice is 8 to 9 μg/g (8, 22), while the LD₅₀ for porfiromycin is 50 μg/g (22). Therefore, 6 μg/g represents a maximally intensive treatment with MC, while much larger doses of porfiromycin can be given safely. In our studies, a larger dose of porfiromycin (20 μg/g), well below the LD₅₀, killed a larger number of the hypoxic tumor cells than could be killed with a nontoxic dose of MC. The combination of 15 Gy of X-rays and 20 μg of porfiromycin per g caused greater cytotoxicity than would be expected if the 2 agents produced additive, independent cytotoxicities in vivo. This suggests that optimizing the combination of porfiro-
Porfiromycin toxicity to hypoxic tumor cells

Porfiromycin and radiation may lead to the development of extremely effective treatment regimens, in which the selective toxicities of each agent for a different microenvironmental subpopulation combine to yield supraadditive toxicities to the cells of solid tumors.

REFERENCES

Porfiromycin as a Bioreductive Alkylating Agent with Selective Toxicity to Hypoxic EMT6 Tumor Cells \textit{in Vivo} and \textit{in Vitro}  

Susan R. Keyes, Sara Rockwell and Alan C. Sartorelli  