Effects of cytokine-induced macrophages on the response of tumor cells to banoxantrone (AQ4N)

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Abstract
Tumor-associated macrophages (TAMs) are found in many solid tumors and have often been shown to accumulate in the hypoxic regions surrounding areas of necrosis. TAMs are the major site of expression of nitric oxide synthase (NOS), a heme-containing homodimeric enzyme consisting of oxygenase and reductase domains. The latter has a high degree of sequence homology to cytochrome P450 reductase and a functional consequence of this is the ability of NOS, under hypoxic conditions, to activate the bioreductive drugs tirapazamine and RSU1069. Banoxantrone (AQ4N) is a bioreductive prodrug activated in hypoxia by an oxygen-dependent two-electron reductive process to yield the topoisomerase II inhibitor AQ4. A feature of this process is that the final product could potentially show bystander cell killing. Thus, in this study, we investigated the ability of inducible NOS (iNOS)-expressing TAMs to activate AQ4N and elicit toxicity in cocultured human tumor cells. Murine macrophages were induced to overexpress iNOS by treatment with a combination of cytokines, mixed with HT1080 and HCT116 human tumor cells, and the toxicity of AQ4N was determined under aerobic or hypoxic conditions. The aerobic toxicity of AQ4N toward tumor cells was increased in AQ4N metabolism and a substantial increase in tumor cell toxicity, which was dependent on the proportion of macrophages in the culture. This study is the first demonstration of TAM-mediated prodrug activation to result in bystander killing of human tumor cells. [Mol Cancer Ther 2009;8(5):1261–9]

Introduction
Solid tumors are composed not only of malignant cells but also many other nonmalignant cell types such as fibroblasts, endothelial cells, and various infiltrating immune cells (1). Tumor-associated macrophages (TAMs) constitute a significant part of the infiltrating immune cells in many human cancers including ovarian, thyroid, breast, lung, kidney, colon, and melanoma, and the percentages of TAMs are often >50% of the total cell population (2). This high density of TAMs correlates with poor prognosis of disease in >80% of published studies (3).

TAMs have been implicated in many aspects of tumor behavior including invasion, inflammation, matrix remodeling, angiogenesis, and metastasis and have close interactions with tumor cells and vasculature through the production of a variety of mediators (4). One of these mediators is nitric oxide (NO), which is produced by three isoforms of NO synthase [NOS; endothelial, neuronal, and inducible (iNOS)]. In cancer, TAMs are the main site of NOS expression, which is generally inducible by inflammatory stimuli such as IFN-γ and lipopolysaccharides (LPS; refs. 5–7). The iNOS gene can also be up-regulated by hypoxia and IFN-γ through the synergistic interaction between the hypoxia inducible factor-1 and the IFN regulatory factor-1 (8). The expression of iNOS in TAMs has been implicated in tumor progression as well as having tumoricidal effects. NO produced by cytokine-induced macrophages is involved in tumor cell killing (9). Interestingly, however, iNOS expression by TAMs has been shown to improve blood flow (10) and promote angiogenesis, hence contributing to its protumor role in cancer (11).

iNOS is a homodimeric enzyme that has two distinct catalytic domains: a heme-containing NH2-terminal oxygenase domain and a P450-like COOH-terminal reductase domain (NOSR; ref. 12). The latter shares a high degree of sequence homology to NADPH:cytochrome P450 reductase (13). Cytochrome P450 reductase and NOSR have been shown to be involved in the metabolism and activation of Adriamycin (14, 15) and certain bioreductive drugs including tirapazamine (16–18).

Banoxantrone (AQ4N) is a hypoxia-activated prodrug currently in phase I/II clinical trials (19). AQ4N undergoes two sequential 2e– reductions, via the mono N-oxide AQM, to give AQ4, a potent topoisomerase II inhibitor. AQ4 is an oxygen-insensitive persistent cytotoxin that does not undergo redox cycling (20, 21). It has, therefore, been...
hypothesized that AQ4N, once activated (to give AQ4), can diffuse to the surrounding cells and exert bystander effects (20). Metabolic activation of AQ4N has previously been shown to be carried out by various cytochrome P450 isoforms under hypoxic conditions. The precise mechanism of AQ4N reduction by cytochrome P450 is still unknown. However, it has been suggested that the nitrogen-oxygen bond cleavage is facilitated through the interaction of N-oxide with the cytochrome P450 heme center. AQ4N metabolism is sensitive to air possibly because oxygen can outcompete AQ4N for binding to the heme (20).

TAMs accumulate in high density in the poorly vascularized hypoxic areas of the tumors generally in close proximity to tumor cells (22). These hypoxic tumor cells are often significantly less responsive to conventional chemotherapy and radiotherapy than the well-oxygenated cells and the presence of these hypoxic regions correlates with poor prognosis of cancer (23). The purpose of this study was to investigate the potential of exploiting the presence of these iNOS-expressing TAMs in the hypoxic regions of the tumors to enhance response to anticancer therapy. As a first step in evaluating this, we have used a combination of cytokines to induce the expression of iNOS in macrophages in vitro. We have determined the ability of these induced macrophages to reductively activate AQ4N and then measured whether coculturing macrophages with human tumor cells under both well-oxygenated and hypoxic conditions can elicit AQ4N/AQ4-mediated bystander toxicity toward the cancer cells.

**Materials and Methods**

**Drugs and Chemicals**

AQ4N (1,4-bis[2-(dimethylamino)ethyl]amino)-5,8-dihydroxyanthracene-9,10-dione-bis-N-oxide) and AQ4 (1,4-bis[2-(dimethylamino)ethyl]amino)-5,8-dihydroxyanthracene-9,10-dione) were provided by KuDOS Pharmaceuticals. Tirapazamine (3-amino-2,4-benzotriazine-1,4-dioxide; SR4233) was synthesized in-house according to the methodology of Fuchs et al. (24). Recombinant murine IFN-γ was purchased from R&D Systems. All other reagents were of analytical grade and purchased from Sigma.

**Cell Culture**

The mouse macrophage cell line J774.2 was grown in DMEM (Life Technologies) supplemented with 10% FCS (LabTech International) and 2 mmol/L L-glutamine (Invitrogen Life Technologies).

The human cancer cell lines HT1080 and HCT116 were stably transfected with a green fluorescent protein (GFP) gene and a puromycin resistance gene to allow for selection when cocultured with macrophages. The cells were grown in RPMI 1640 supplemented with 10% FCS and 2 mmol/L L-glutamine. Stability of the HT1080 enhanced GFP (EGFP) and HCT116 EGFP cells was frequently monitored by growing the cells in the presence of puromycin (5 μg/mL) and checking fluorescence of the cells using a fluorescence-activated cell sorter (BD Biosciences).

Cells were incubated under aerobic conditions at 37°C in a humidified atmosphere of 95% air/5% CO2. Hypoxic incubations were carried out at <0.001% O2 in a catalyst-induced hypoxic environment (Bactron Anaerobic Chamber, Sheldon Manufacturing).

**Induction of iNOS Expression in Macrophages Using Cytokines**

Macrophages were plated at a density of 3 × 10⁶ cells in 6 cm culture plates in serum-free medium for 24 h before they were exposed to a combination of IFN-γ (25 ng/mL) and different concentrations of LPS (10, 25, 50, and 100 μg/mL) for 24 h. Following treatment, the cytokines were washed out and serum was returned into the medium. The cells were reincubated for another 24 h at which point the culture medium was collected and the cells were harvested for subsequent analysis.

**Nitrite Analysis**

To determine the effect of cytokines on iNOS induction in macrophages, we used the Griess reaction as described by Dong et al. (25) to quantify the amount of nitrite accumulated in the medium of cytokine-induced cells as a surrogate measure of NO production.

**Protein Determination**

The amount of total protein in the cell lysates was determined by the Pierce bicinchoninic acid assay (26) with bovine serum albumin as a protein standard.

**iNOSR Activity**

Following cytokine treatment of the J774.2 cells, the NOSR activity was determined spectrophotometrically in cell lysates as the NADPH-dependent reduction of cytochrome c as described previously (17).

**Western Blot Analysis**

Proteins were resolved by electrophoresis on a 7% SDS-polyacrylamide gel and electrophotorethically transferred to a protein binding membrane. Samples were loaded based on equal amounts of protein. As primary antibodies, the murine iNOS (1:5,000 in TBS/3% bovine serum albumin) antibody (Biomol) or human iNOS (1:1,000 in TBS/3% bovine serum albumin) antibody (Zymed Laboratories) was added and incubated for 1 h at room temperature. For protein detection, a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:12,000 in PBS-Tween 20/5% skimmed milk) was used. Signal detection was done by an immunoperoxidase-based ECL technique (Pierce) according to the manufacturer’s protocol.

**High-Performance Liquid Chromatography Analysis of AQ4N and Its Metabolites**

Incubations of cell lysates with AQ4N were carried out under nitrogen at 37°C in 4 mL amber glass vials (Chromaco) sealed with subaseal (Aldrich). Hypoxic conditions were generated using zero-grade nitrogen gas passed through an oxy-trap (Alltech). Metabolism was initiated by addition of NADPH and samples were removed for analysis at various times thereafter. Chromatographic analysis of AQ4N was adapted from the method of Swaine et al. (27). A Hichrom RPB column (25 cm × 4.6 mm i.d.; serial no. HIRPB-6985; Hichrom) was used for the isocratic separation of the metabolites at a flow rate of 1.5 mL/min. UV detection of column
eluates was monitored at 242 nm. The mobile phase consisted of acetonitrile-ammonium formate buffer (0.05 mol/L; 20:80, v/v), with the final pH adjusted to 3.5 with formic acid. Calibration standards were prepared from stock solutions in DMSO and diluted appropriately in initial mobile phase. Approximate retention times under these conditions were 13.3, 10.2, and 8.1 min for AQ4N, AQM, and AQ4, respectively. Concentrations of metabolites were calculated from peak height ratios and compared with calibration curves prepared by spiking inactivated lysate preparations with known amounts of metabolite. The values were then corrected for protein content and expressed as pmol metabolite formed/min/mg protein. Mitoxantrone was used as an internal standard.

**Drug Sensitivity by Colony Formation Assay**

HT1080 EGFP cells (1 × 10^5) were cultured with equal numbers of cytokine-induced macrophages in 6-well plates. The cells were cultured in medium containing 100 μmol/L N-methyl-l-arginine (NMLA), a NOS oxygenase inhibitor, to preclude any contribution to the observed toxicity by NO. The coculture was allowed to attach overnight in air or hypoxia. The cells were then exposed to different concentrations of AQ4N for 90 min. The drug was removed, the cells were trypsinized, and the GFP-positive cells counted (not sorted) using fluorescence microscopy and seeded for colony formation (6-10 days) in medium containing NMLA and puromycin to allow selection for GFP-positive cancer cells. After this period, the medium was removed and the colonies were fixed in 70% ethanol and stained with methylene blue to facilitate counting of colonies (≥50 cells). Clonogenic survival was calculated for each drug dose after correcting for plating efficiency. The IC_{50} values were calculated from clonogenic survival curves as the concentration of the drug required to cause 90% cell kill.

**Drug Sensitivity by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Proliferation Assay**

Dose-response curves were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay, which is based on the ability of viable cells to convert a soluble tetrazolium salt, MTT, into purple formazan crystals. Relevant numbers of HT1080 EGFP and HCT116 EGFP cells were seeded in 96-well plates with the following percentages of cytokine-induced macrophages (0, 1%, 5%, 10%, 25%, 50%, and 80%) at 37°C under either aerobic or hypoxic conditions 3 h before exposure to drug in medium containing NMLA (100 μmol/L). The cells were then exposed to different concentrations of AQ4N or AQ4 for 90 min. In these experiments, tirapazamine was used as a positive control for bioreductive drug toxicity and exposure to this drug was for 3 h. Subsequently, the drugs were removed and the cells were allowed to grow in medium containing puromycin (5 μg/mL) and NMLA for 4 days before the MTT was added to the wells. The IC_{50} values were calculated from dose-response curves as the concentrations of the drug required to cause a 50% reduction in absorbance compared with the untreated controls. The IC_{50} values quoted are the means of at least three independent experiments. Hypoxic cytotoxicity ratios were determined as the ratio aerobic IC_{50}/hypoxic IC_{50} for each drug and cell line tested.

**Cell Cycle Analysis**

HT1080 EGFP cells were seeded at the required density with an equal number of cytokine-induced macrophages in medium containing NMLA and allowed to attach in air before they were transferred to hypoxia for an 18 h exposure. The mixed-cell population was collected in serum-free medium containing 25 mmol/L HEPES. The two cell types were separated according to their GFP profile in a fluorescence-activated cell sorter. In a parallel experiment, tumor cells were cocultured with an equal number of cytokine-induced macrophages under hypoxic conditions in medium containing NMLA. After 3 h of coculture, puromycin and NMLA were added to the medium and the cells were left to grow in air for 4 days before collection for cell cycle analysis. Monocultures of HT1080 EGFP in each experiment were used as controls.

The collected HT1080 EGFP cells were fixed in ice cold 70% (v/v) ethanol solution while vortexing and the fixed cells were stored at 4°C overnight. Before further analysis, the cells were centrifuged at 300 g for 5 min and the 70% (v/v) ethanol was removed. Cells were washed with PBS before being resuspended in 1 ml PBS containing 50 μg/mL propidium iodide and 250 μg/mL RNase A [10 mmol/L Tris (pH 7.5) and 15 mmol/L NaCl] for 30 min at room temperature in the dark. The cells were finally repelleted and resuspended in PBS for immediate analysis of the fixed propidium iodide-stained cells using flow cytometry.

**Statistical Analysis**

All data shown are from at least three independent experiments. Data are expressed as mean ± SD for the indicated numbers of experiments. Statistical analysis was done using Student’s t test between two experimental groups.

**Results**

**Induction of iNOS in Macrophages**

Significant induction of iNOSR activity in the murine macrophage cells was achieved 24 h after incubation with a combination of IFN-γ and LPS reaching a maximal level at a combination of IFN-γ (25 ng/mL) and LPS (25 μg/mL; Fig. 1A). The total cellular reductase activity increased from 13.8 nmol/min/mg protein in control macrophages to a maximum activity of about 38.5 nmol/min/mg protein in the induced samples. Under nonstimulatory conditions, macrophages generate very low levels of NO shown by the production of 2.6 μmol/L nitrite as measured by Griess assay. However, exposure of macrophages to the combination of LPS and IFN-γ for 24 h followed by an additional 24 h in culture triggered the release of significantly higher levels of NO from the induced macrophages (340 μmol/L nitrite). As shown in Fig. 1A, the pattern of NO generation by the macrophages follows the induction in cellular reductase levels as nitrite accumulation also reached a maximal level at the cytokine combination of IFN-γ (25 ng/mL) and LPS (25 μg/mL).
Therefore, it can be concluded that the difference in the cellular reductase activity between control and cytokine-induced macrophages corresponds to the induction of the iNOS gene and therefore the iNOSR activity.

We also analyzed the induced macrophages for iNOS protein expression by Western blotting. The assay clearly shows the induction of iNOS protein in macrophages induced with IFN-γ (25 ng/mL) and LPS (25 μg/mL) compared with control (Fig. 1B).

The concentration of cytokines (LPS 25 μg/mL and IFN-γ 25 ng/mL) that achieved maximal induction of iNOS activity in macrophages did not affect cell viability as determined by the MTT assay (data not shown). Therefore, this concentration of cytokines was selected to induce the macrophages in subsequent studies.

**Activation of AQ4N by Cytokine-Induced Macrophages**

We sought to compare the ability of control and cytokine-induced macrophages to metabolize AQ4N under hypoxic conditions. Lysates (2-3 mg/mL protein) of control and cytokine-induced macrophages were exposed to 20 μmol/L AQ4N under nitrogen for 60 min and then analyzed for the presence of the metabolites AQM and AQ4 by high-performance liquid chromatography (Table 1). We observed a 2.5- and 2.4-fold increase in the rate of formation of AQM and AQ4 respectively by the cytokine-induced macrophages in comparison with control macrophages, suggesting that the induction of iNOS protein in the macrophages resulted in an increased rate of AQ4N metabolites formation in hypoxia.

**Toxicity of AQ4N and AQ4 Toward Cytokine-Induced Macrophages and Human Tumor Cells**

We showed that cytokine-induced macrophages can metabolize AQ4N to its toxic metabolites; hence, they could act as produg activating cells in a tumor microenvironment. Before investigating the bystander properties of AQ4N using a coculture setting, we first sought to assess the aerobic and hypoxic toxicity of AQ4N to induced-macrophages and the human tumor cells (HT1080 and HCT116) in a monoculture setting (Table 2).

The cytokine-induced macrophages showed the highest HCR value (ratio of IC50 values under aerobic and hypoxic conditions) of 10 with an aerobic IC50 of 40.0 μmol/L and a hypoxic IC50 of 3.9 μmol/L, suggesting the high rate of drug metabolism under conditions of low oxygen as a possible factor for their increased sensitivity to AQ4N under hypoxic conditions. HT1080 EGFP cells showed the highest sensitivity to AQ4N compared with the other cell lines under both aerobic and hypoxic conditions (IC50 values of 1.6 μmol/L in air and 0.4 μmol/L in hypoxia). HCT116 EGFP cells had the lowest sensitivity to AQ4N with a hypoxic IC50 of 28.9 μmol/L, which is ∼72 and 7.4 times higher than the hypoxic IC50 observed in HT1080 EGFP cells and the cytokine-induced macrophages, respectively. The HCR value for HCT116 EGFP cell line was ∼1, suggesting a lack of ability to metabolize AQ4N or an intrinsic resistance to the toxic metabolites as possible factors contributing to the low differential toxicity of AQ4N in this cell line.

We next examined the toxicity of AQ4 to the human cancer cells by exposing the cells to the drug for 90 min under...
aerobic conditions. Cytotoxicity was measured using MTT assay (Table 2).

The HCT116 EGFP cells had an IC50 of 62 nmol/L for AQ4, which represents ~3-fold resistance to the metabolite compared with HT1080 EGFP cells. Although this may partially explain the lower sensitivity of the HCT116 EGFP cells to AQ4N, it may not account for its low differential toxicity (HCR = 1).

Cytokine-Induced Macrophages Enhance AQ4N Toxicity to the Cancer Cells in a Coculture Model

AQ4N is a nontoxic produg that is selectively activated in hypoxic cells to the stable, DNA-affinic cytotoxin, AQ4. It has been suggested that AQ4 is available to diffuse to the surrounding tumor cells possibly allowing a "bystander effect" (20).

To assess whether AQ4N has bystander effects, we cocultured equal numbers of the cytokine-induced macrophages (prodrug activating cells) with the tumor cell line HT1080 EGFP (bystander target cells) and measured the effect of AQ4N-induced toxicity to the cancer cells using the clonogenic survival assay.

Figure 2A shows that coculture of HT1080 EGFP cells with the cytokine-induced macrophages is associated with an enhanced sensitivity of the cancer cells to AQ4N under hypoxic conditions only. We observed ~2-fold decrease in the hypoxic IC50 of the HT1080 EGFP cells in cultures comprising 50% cytokine-induced macrophages compared with monocultures. The toxicity of AQ4N to tumor cells under aerobic conditions was not affected by the presence of cytokine-induced macrophages in the cultures.

The efficiency of selection for HT1080 EGFP colonies was straightforward because macrophages are not able to form colonies on plastic Petri dishes. The cloning efficiencies of the cancer cell line in monocultures and cocultures were comparable, suggesting that the cytokine-induced macrophages were not toxic to the human cells.

We then evaluated the dependence of HCR for the HT1080 EGFP cells on the concentration of activating macrophages in the coculture. For these experiments, we used the MTT assay. We observed that the presence of cytokine-induced macrophages in coculture, under aerobic conditions, did not affect AQ4N-induced toxicity to the cancer cells (data not shown). Under hypoxic conditions, however, there was a concentration (of cytokine-induced macrophages)-dependent decrease in AQ4N IC50 (Fig. 2B).

### Table 2. AQ4N and AQ4 cytotoxicity (90 min exposure) against the cytokine-induced macrophages and the human cancer cells in air and hypoxia as determined by MTT assay

<table>
<thead>
<tr>
<th>Cell line</th>
<th>AQ4N (IC50)*</th>
<th>AQ4 (IC50)*</th>
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<tbody>
<tr>
<td></td>
<td>Air (μmol/L)</td>
<td>Hypoxia (μmol/L)</td>
</tr>
<tr>
<td>Induced macrophage</td>
<td>40.0 ± 10.5</td>
<td>3.9 ± 3.8</td>
</tr>
<tr>
<td>HT1080 EGFP</td>
<td>1.6 ± 0.6</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>HCT116 EGFP</td>
<td>28.6 ± 16.5</td>
<td>28.9 ± 11.5</td>
</tr>
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NOTE: HCR is defined by the aerobic-to-hypoxic IC50 ratio.
IC50 values are determined as the drug concentrations required to achieve 50% cell kill compared with control. HCR values were obtained by dividing the aerobic IC50 of the drug by the hypoxic IC50.

*Mean ± SD of three independent experiments done in triplicate. ND, determined.

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**Figure 2.** A, clonogenic survival curves for HT1080 EGFP exposed to AQ4N under aerobic or hypoxic conditions when in monoculture or as cocultures consisting of 50% cytokine-induced macrophages. Data represent the surviving fraction relative to that of control untreated cells. Mean ± SD obtained from duplicate plates in three independent experiments. B, HCR values for HT1080 EGFP cells cocultured with increasing numbers of cytokine-induced macrophages. Toxicity was determined by MTT assay and HCR values were obtained by dividing the aerobic IC50 of the drug by the hypoxic IC50 for each condition. Points, average from three independent experiments; bars, SD. *, P < 0.05; **, P < 0.001, significantly different from control.
Macrophages, the drug concentrations required to cause 50% cell kill in HT1080 EGFP cells decreased by 50% compared with control. In cultures consisting of 10%, 50%, and 80% macrophages, the HCR increased from a value of 3.6 in monoculture to values of 9.6, 13.8, and 49.1, which correspond to ~2.6-, 3.8-, and 13.6-fold decrease in the hypoxic IC50, respectively. This suggests activation of AQ4N to AQ4 by the iNOS-expressing macrophages and the subsequent diffusion of the metabolite to the cancer cells is the causative mechanism of the increased toxicity.

We also assessed this AQ4N-induced toxicity in the more resistant HCT116 EGFP cells. As observed with the HT1080 EGFP cells, the aerobic toxicity of AQ4N was not enhanced on coculture with the iNOS-expressing macrophages. However, under hypoxic conditions, we again observed a significant enhancement in drug toxicity in cocultures consisting of 80% macrophages where the HCR value increased by 10-fold (data not shown). In these assays, the growth rate of the tumor cells was not affected after coculture with the cytokine-induced macrophages. Furthermore, the duration of exposure to puromycin following drug treatment (5 μg/mL for 4 days) was sufficient to kill all the murine macrophages (before giving MTT). This ensured that the absorbance of the formazan product was derived from the tumor cells only (data not shown).

Cytokine-Induced Macrophages Do Not Enhance Cancer Cells Sensitivity to Tirapazamine

Tirapazamine was chosen as a negative control in this study because its one-electron reduced, toxic, free-radical product is short-lived and highly reactive and therefore very unlikely to exhibit bystander cell killing (28). Cocultures consisting of 80% cytokine-induced macrophages were exposed to different concentrations of tirapazamine under both aerobic and hypoxic conditions and toxicity of the drug to the cocultured HT1080 EGFP cells was assessed using the MTT assay. We found that the IC50 of tirapazamine in monocultures was comparable with the IC50 observed in cocultures (Table 3). Therefore, in the presence of iNOS-expressing macrophages, tirapazamine-induced toxicity to the cancer cells was not affected under both aerobic and hypoxic conditions. This confirms that, using a coculture model of cytokine-induced macrophages and cancer cells, we are able to show the bystander properties of AQ4N/AQ4.

Cytokine-Induced Macrophages Did Not Affect Cell Cycle Distribution of the Cancer Cells

To determine whether cell cycle changes could account for the enhanced toxicity of AQ4N to the tumor cells in the coculture model, cell cycle analysis of the tumor cells was done. For HT1080 EGFP tumor cells held in air in monoculture, the cell cycle distribution was 48.6 ± 2.0%, 23.2 ± 0.7%, and 23.1 ± 0.8% for G1, S, and G2-M phase cells, respectively, which was similar to the distribution of cells in coculture (52.7 ± 1.2%, 22.0 ± 2.3%, and 21.0 ± 0.7%, respectively). After 18 h of coculture in hypoxia, the percentage of HT1080 EGFP cells in G1, S, and G2-M phases were 59.8 ± 0.3%, 12.9 ± 2.1%, and 20.6 ± 0.9%, respectively. In monoculture, the distribution was 67.5 ± 1.1%, 23.2 ± 0.7%, and 16.6 ± 0.6%, respectively. Thus, it appears that the hypoxic exposure causes some cell cycle perturbation, but this is not affected by the presence of the macrophages.

Effect of Cytokine-Induced Macrophages on iNOS Gene Expression in the Tumor Cells

To ensure that the enhancements we have observed were solely due to macrophage-derived NOS activation, we analyzed, using Western blotting, the level of iNOS protein expression in HT1080 EGFP cells 18 h after coculture with the cytokine-induced macrophages. Figure 3 clearly shows that expression of the iNOS protein is not induced in the tumor cells. Hence, the enhanced AQ4N toxicity previously observed in our coculture studies is

<table>
<thead>
<tr>
<th>Condition</th>
<th>Tirapazamine (IC50)</th>
<th>Differential toxicity (HCR)</th>
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<tbody>
<tr>
<td>Monoculture</td>
<td>271.5 ± 98.3</td>
<td>20.1 ± 22.3</td>
</tr>
<tr>
<td>Coculture</td>
<td>239.4 ± 119.7</td>
<td>16.8 ± 19.0</td>
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NOTE: HCR values were obtained by dividing the aerobic IC50 of the drug by the hypoxic IC50. HT1080 EGFP cells in monocultures and cocultures were exposed to tirapazamine for 3 h in air or hypoxia.

*Mean ± SD of three independent experiments done in triplicate. IC50 values are determined as the drug concentrations required to achieve 50% cell kill compared with control.

Figure 3. Western blot representing iNOS protein expression in HT1080 EGFP cells from monocultures (lane 1) and cocultures (lane 2) under hypoxic conditions. HT1080 cells transfected with iNOS gene were used as a positive control (lane 3). Actin is shown to verify equal loading.
not due to enhanced metabolism of AQ4N by the tumor cells themselves.

**Discussion**

In the current study, we clearly show that cytokine-induced macrophages can activate AQ4N to AQ4, which is then able to diffuse to the surrounding tumor cells and exert bystander cell killing. We have used this coculture model as it mimics, to some extent, the physiologic microenvironment of tumors. It is believed that macrophages are recruited to the hypoxic region of the tumor under the influence of chemotactants released by the hypoxic cells within the tumor and the increasing gradient of necrotic cell debris, although they may also travel randomly within tumors until they become immobilized in the hypoxic regions (29). TAMs also accumulate in hypoxic regions of small micrometastasis (30, 31). These hypoxic regions of the tumors are the most resistant to conventional chemotherapy and radiotherapy (23). We hypothesized that the natural accumulation of TAMs in these hypoxic regions and their expression of iNOS could be exploited to enhance the metabolism of hypoxia-activated prodrugs with bystander properties to improve their antitumor effects.

We selected mouse macrophages because they are able to respond to a combination of IFN-γ and LPS, via an IFN-γ response element and nuclear factor-κB element in the promoter region of the iNOS gene, to readily induce up-regulation of the iNOS protein (32). Our data show that cytokine-induced murine macrophages express high levels of iNOS protein as detected by Western blotting. The NOSR activity in those macrophages also increased significantly 24 h after exposure to cytokines, confirming the induction of a functionally active iNOS enzyme.

We have also shown that our coculture model of iNOS-expressing macrophages and cancer cells is useful in investigating the bystander effects of prodrugs that are activated by inducible NOSR. Firstly, we were able to use these iNOS-expressing macrophages as the prodrug activating cells because our high-performance liquid chromatography studies showed that AQ4N metabolism to its toxic metabolites was significantly enhanced in hypoxia when the murine macrophages were activated by cytokines to overexpress iNOS. Analysis by high-performance liquid chromatography allows the determination of the amount of drug that is converted into the two-electron reduction product AQM and the four-electron end product AQ4. Although suggested by Patterson and McKeown (20) to suggest that AQ4N could have a bystander effect. Therefore, we sought to assess and quantify for the first time the bystander effects of AQ4N in vitro using a coculture model of cytokine-induced macrophages and human cancer cells.

Under hypoxic conditions, percentage killing of the HT1080 cells was associated with the percentage of cytokine-induced macrophages in the coculture with significantly enhanced toxicity observed in cocultures consisting of as little as 5% macrophages.

The cytokine-induced macrophages were more resistant to AQ4N than the HT1080 cells, which allows the macrophages to act as a metabolic center for prodrug activation. Tirapazamine is another bioreductive drug selectively activated by iNOS under hypoxic conditions to give highly toxic metabolites (17). Here, we show that coculturing the cancer cells with the iNOS-expressing macrophages did not enhance the toxicity of tirapazamine to cancer cells under both hypoxic and aerobic conditions. This is most likely because the tirapazamine radical is short-lived and highly reactive, which prevents the drug from exerting any bystander effects (17, 28). However, it also highlights the potential value and utility of AQ4N, which does show these effects.

Previous published data have shown that macrophage density has a prognostic significance in a variety of human tumors. It has been shown to correlate with poor prognosis in carcinomas of the breast (37, 38), cervix (39, 40), and bladder (41), confirming to some extent the protumorigenic role of macrophages. On the other hand, macrophages in stomach cancer were associated with good prognosis of the disease (42), and there has been varying results with lung cancer (43, 44). Two studies on 26 and 30 patients, respectively, showed that low infiltration of macrophages correlated with more advanced colorectal cancer (45, 46). A recent study indicated that a dense macrophage infiltration at the tumor front positively influenced prognosis in colon cancer (47). Hence, TAMs are an important feature of cancer and their level of infiltration into tumors has an important prognostic value.
TAMs are the main site of iNOS expression in human breast cancer as shown by Thomsen et al. (7). In this type of cancer, we observed a 5-fold increase in total NOS activity in breast tumor biopsies compared with normal breast tissue (48). iNOS expression in human breast tumor models was also confirmed by immunohistochemical analysis and appeared to be localized primarily in areas between viable and necrotic regions of the tumor (an area that is presumably hypoxic; ref. 49). Therefore, information on the level of macrophage infiltration into the hypoxic regions and the level of iNOS expression is a potential endogenous marker for tumor response to AQ4N and other bioreductive drugs with bystander properties. Our findings also provide the basis for including AQ4N in a NOS-mediated GDEPT therapy, although this approach is currently restricted by the inability to control the level of iNOS gene expression in the tumors, which is crucial to the fate of the tumor as high levels of NO can inhibit tumor growth and metastasis, whereas low levels can protect the cells from apoptosis and hence promote tumor growth (50).

In conclusion, our study is the first demonstration of the utility of the macrophage/cancer cells coculture model in quantifying the bystander effects of AQ4N. Our model exploits the environmental heterogeneity of the tumors by targeting the infiltrated iNOS-expressing macrophages that reside in the hypoxic regions of the tumors. We believe that the stable nature of AQ4N metabolite enables it to induce a bystander cell killing effect on proximate tumor regions. This approach may offer several advantages in curative chemotherapy, including significant killing of the surrounding tumor cells with clinically better-tolerated doses of prodrugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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