9

In Vitro Testing of Chemosensitivity in Physiological Hypoxia

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Summary

Highly aggressive, rapidly growing tumors are often hypoxic, owing to an inadequate supply relative to consumption of oxygen (O_2) in the expanding tumor mass, or growth in tissues with physiologically low O_2 concentrations (such as bone marrow). Selection of tumor cells that can grow or survive under hypoxic conditions appears from both experimental and clinical studies to impact tumor progression, response to therapy, and to increase resistance to radiation and to certain cytotoxic drugs. Therefore, the predictive value of preclinical testing of anticancer agents in cell culture might be improved by conducting testing in conditions of physiological hypoxia. We review the impact of hypoxia on anticancer drug cytotoxicity and the methods used in our laboratory to asses the cytotoxic activity of single antineoplastic drugs under conditions of physiological hypoxia.

Key Words

Tumor hypoxia; drug resistance; hypoxia-targeted therapy; cytotoxicity assay; digital image microscopy.

1. Introduction

Solid tumors often have areas in which circulation is compromised because of structurally disorganized blood vessels and tumor cells that grow faster than the developing tumor capillary network ***(1,2)***. The fraction of solid tumors that are hypoxic can vary from 0.2 to >50% ***(3,4)***. The degree of hypoxia in tumors is highly variable, with the Po_2 generally <10–30 mmHg (1–3% O_2), in contrast to a Po_2 of 50–80 mmHg in most normal tissues ***(4)*** **(Table 1)**. The microenvironment found in hypoxic tumors leads to low extracellular pH from lactic acid, low glucose, genomic instability, and selective pressure to adapt and survive ***(5,6)***. In addition, hypoxia is associated with local increases in tumor interstitial

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Table 1
O_2 Tensions in Various Tissues, Tumors, and Cell Cultures

	Po_2 (mmHg)[a]	References
Normal tissues		
Murine brain	60	Unpublished data
Murine muscle	42	Unpublished data
Bone marrow (children)	44	***28***
Bone marrow (adult)	40–50	***21***
Normal liver	~55	***29***
Normal breast tissue	65	***30,31***
Subcutaneous tissue	38	***20,32,33***
Normal cervix	48	***33***
Normal head and neck tissue	43	***33***
Tumors		
Breast carcinoma	28	***30***
Solid tumors	≤2.5	***11,20,34***
Murine Fsall fibrosarcoma	≤5	***33***
Cancer of cervix	≤12	***26,33***
Head and neck cancers	≤10	***20,32***
Soft-tissue sarcomas	≤10 and ≤22	***1,20***
Neuroblastoma xenograft in athymic (nu/nu) mouse	7	Unpublished data
Tissue Culture		
RPMI-1640 medium with 10% FBS incubated at 2% O_2	12.6	Unpublished data
RPMI-1640 medium with 10% FBS incubated at 5% O_2	47	Unpublished data
RPMI-1640 medium with 10% FBS incubated at 20% O_2	149	Unpublished data

[a]Po_2 values vary from study to study based on the methods used for Po_2 level measurements. However, these relatively constant values are from studies done using similar methods to measure Po_2 levels in normal and tumor tissues (computerized Po_2 and polaragraphic electrodes). The % O_2 was calculated based on correlations between mm Hg and % O_2, done by Stone ***(31)***. FBS, fetal bovine serum.

fluid leading to microthrombi and increased blood viscosity ***(6)***. This microenvironment has prognostic implications, because cells in hypoxic areas are less vulnerable to ionizing radiation and cytotoxic drugs, and tumors with substantial hypoxia metastasize more efficiently ***(1,2)***. Hypoxia is a prognostic variable for unfavorable outcome, because it provides a mechanism by which tumors can selectively promote a more aggressive phenotype, recruit a nutrient supply,

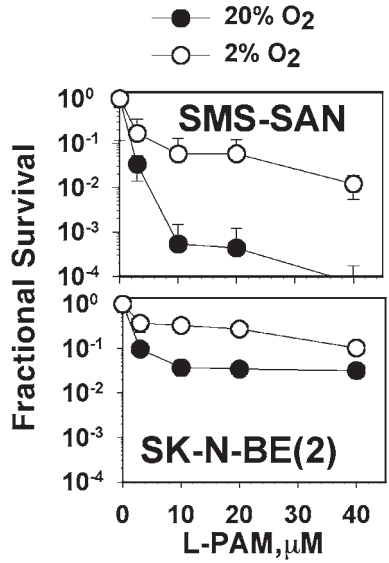

Fig. 1. Hypoxia (2% O_2) significantly antagonizes L-PAM cytotoxicity. The effect is most pronounced in the p53-functional SMS-SAN cell line (established at diagnosis) but is also seen in the *TP53*-mutated multidrug resistant cell line, SK-N-BE(2), estabalished at relapse after chemotherapy ($p < 0.01$). Similar results were obtained with other neuroblastoma cell lines.

and may promote essential metabolic adaptations that improve tumor survival ***(1,2,7)***. Hypoxia is also considered to protect cells in solid tumors from chemotherapeutic agents ***(3,4,7–9)***, although there are only a limited number of direct studies supporting this concept, mostly with doxorubicin ***(9,10)*** and methotrexate ***(10)***. This induced resistance can be explained by decreased drug concentrations, because of limited drug penetration into tumor masses; decreased drug activity in areas where tumor cells are slowly growing or nonproliferating due to hypoxia and the associated alterations in nutrient supply and utilization; and direct antagonism of drug action by hypoxia and the associated acidosis ***(11)***.

Many chemotherapeutic agents are dependent on cellular oxygenation for maximal efficacy. Cytotoxic alkylating agents, such as the nitrogen mustard alkylating agent melphalan (L-PAM), are a class of chemotherapeutic drugs that act by transferring alkyl groups to DNA during cell division. Following this, the DNA strand breaks or crosslinking of the two strands occurs, preventing subsequent DNA synthesis ***(12)***. Teicher et al. ***(13)*** showed that tumor cells grown in normoxic conditions were more sensitive to L-PAM than in hypoxic conditions, and we have observed similar results **(Fig. 1)**. Under hypoxic

conditions, alkylating agents may have less activity for a variety of reasons, including an increase in intracellular glutathione, which may compete with the target DNA for alkylation ***(11,14)***.

Hypoxia also causes cells to slowly cycle and induces pre-DNA-synthetic (pre-S-phase) arrest in cells ***(1,7)***. Therefore alkylating agents have reduced efficacy against slow cycling tumor cells under hypoxic conditions, because they have increased activity in apoptosis induction during highly active cell proliferation ***(14)***. Other drugs directly affected by hypoxia include the podophyllotoxin derivative etoposide ***(12,15)***, presumably due to free-radical scavengers, dehydrogenase inhibitors, and dehydrogenase substrates, which prevent the formation of single-strand breaks and decrease the cytotoxic effect of etoposide ***(12)***. DNA-damaging chemotherapeutic agents may also have compromised function due to increased activity of DNA repair enzymes under hypoxic conditions ***(16)***.

An example of a drug with significant antagonism by hypoxia is the glutathione (GSH) synthesis inhibitor buthionine sulfoximine (BSO), which as a single agent was highly cytotoxic against a wide range of neuroblastoma cell lines under standard cell culture conditions (20% O_2) ***(17)***. Physiological hypoxia as found in solid tumors (2% O_2) abrogates the cytotoxic effect of BSO for neuroblastoma by diminishing the increase in reactive oxygen species (ROS) caused by BSO ***(18)***, and that antagonism appeared to be more pronounced in cell lines lacking functional p53 ***(18)***.

The difference between hypoxic cancer cells and normal cells could give researchers a basis on which to design drugs or drug combinations. Cytotoxic drugs active in hypoxia are currently under investigation ***(19)***, including the bioreductive agent tirapazamine (TPZ) ***(2)***. In the presence of oxygen, TPZ is a stable, nontoxic parent molecule. However, TPZ is transformed by intracellular reductases into a highly reactive and toxic radical in the presence of hypoxia ***(2)***. Because TPZ is relatively ineffective at killing aerobic cells, this agent alone is not well studied ***(2)***. We have shown that TPZ synergistically reversed inhibition of BSO-mediated cytotoxicity in hypoxia for human neuroblastoma cell lines, by increasing the formation of ROS, decreasing mitochondrial membrane potential ($\Delta\psi_m$), depleting GSH, and increasing apoptosis ***(18)***. These data suggest that combining BSO with TPZ could have clinical activity against neuroblastoma in hypoxic sites ***(18)***.

Oxygen levels are typically quite heterogeneous, both among patients and within individual tumors. The oxygenation status of tissues has primarily been measured using either polarographic oxygen electrodes or biochemical techniques that rely on antibody detection of nitroimidazole-based adducts in hypoxic tissue ***(20)***. A comparison of tissue oxygenation levels to clinical tumor behavior suggests an advantage for tumors in hypoxia. At diagnosis, neuro-

blastoma most commonly develops in relative hypoxic sites (e.g., the Po_2 in bone marrow is 40–50 mmHg) ***(21)*** **(Table 1)** such as the retroperitoneum, bone, and bone marrow. By contrast, recurrent neuroblastomas more commonly metastasizes to high-oxygen-tension sites such as brain and lungs. Therefore, low oxygen tension could provide a sanctuary site for tumors leading to selection for oxygen tolerance and drug resistance ***(22)***.

Multiple lines of evidence point toward profound differences in the behavior of tumor cells under conditions of high levels of oxygen relative to conditions of physiological hypoxia. This suggests that determination of anticancer drug activity in standard culture conditions (i.e., 20% O_2, which is 10-fold greater than the O_2 levels of many tumors, and 4- to 5-fold greater than bone marrow) may cause an artifactually high estimation of drug activity. For that reason, it is important to validate drug activity in hypoxic conditions, and such an approach may increase the predictive value of cell culture drug testing. Here, we describe methods used to evaluate cytotoxic activity of various drugs and drug combinations in hypoxic conditions.

2. Materials

1. Fluorescein diacetate (FDA) (Sigma, St. Louis, MO): 1 mg/mL solution prepared in dimethyl sulfoxide, aliquoted into 1.5-mL Eppendorf tubes, and kept at –20°C in the dark. Avoid repeated thawing and refreezing.
2. Eosin Y: 1% stock solution (w/v) prepared in 0.9% NaCl and kept in an amber bottle at room temperature.
3. DIMSCAN system (*see* Chapter 12). This system consists of an inverted microscope, a stepper motor scanning stage, a stage controller, a charge-coupled device (CCD) camera, and a Pentium 4 microcomputer (Microsoft Windows 2000) running the main application software (DIMSCAN 3.0, developed at Childrens Hospital Los Angeles, CA), which controls stage movement and processes CCD camera images ***(23,24)***. An inverted microscope, Olympus IX50, is equipped with a 103-W mercury vapor lamp (HBO®-103 W/2), optical filters (Omega Optical [Woburn, MA] XF22 filter set for FDA or BCECF [excitation: 490 nm; emission: 525 nm] and Omega Optical XF05 filter set for Hoechst 33342 [excitation: 345 nm; emission: 475 nm]) and ×4 high N.A. objective lens. It is also equipped with a motorized Prior Pro Scan stage with two stepper motors for stage movements in the horizontal plane (x and y) and one stepper motor for focusing (z-axis). The stage controller communicates with the computer through a serial port. A Qimaging Microimager II CCD camera is attached to the standard trinocular head with an 80/20 beam splitter. Maximum resolution of the camera CCD chip is 1024/768 pixels, and the camera has internal cooling, enabling long-term use without degrading image quality. The camera is connected to the PC through IEEE1394 FireWire interface, which enables a high-rate data transfer to the PC.
4. Data analyzer software. Following incubation with drugs and scanning of microwell plates by DIMSCAN, the Data Analyzer software (developed at Childrens Hospital

Los Angeles, CA) calculates fractions of affected cells (F_a) ($F_a = 1 - RF_{drug}$ / *RF* control), survival fractions (calculated as RF_{drug} / *RF* control), standard deviations (SDs), confidence levels, and standard errors using the relative fluorescence (*RF*) values obtained during the DIMSCAN. SigmaPlot (Jandell, San Rafael, CA) can be used to create dose-response graphs, and Excel (Microsoft Office) to determine fractions of affected cells, survival fractions, and SDs.

5. Nitrogen cylinders (Praxair, Danbury, CT): Refrigerated liquid nitrogen cylinders containing 160 L (3690 ft^3) liquid nitrogen at intracylinder pressure of 230 psi deliver nitrogen gas to the incubators at 10–15 psi. Custom gas mixtures with 0.6%–5% oxygen, 5% CO_2, and the balance N_2 are provided in cylinders with 25 ft^3 under 2000 psi. Nitrogen cylinders must be equipped with proper regulators in order to deliver nitrogen or a mixture of gases at a desirable pounds per square inch (psi).
6. Tissue culture incubators for O_2 level control: There are three major approaches to maintaining a designated hypoxic environment inside incubators: using incubators with an integrated O_2 control system **(Fig. 2)**, placing a sealed modular incubation chamber with a separate CO_2 and O_2 control system inside a standard host incubator **(Fig. 3)**, and placing inside the host incubator a hermetically sealed chamber that has been "flushed" with the appropriate O_2 concentration **(Fig. 4)**. We present here examples of these three different approaches.
   a. CO_2- and O_2-Controlled water-jacketed incubator. The following applies to the Model 3110 Incubator from ThermoForma (Marietta, OH). Other vendors, such as Sanyo (Itasca, IL), also make O_2-regulated incubators. The Forma 3110 water-jacketed incubator has separate regulators for O_2 and for CO_2, allowing the user to define the exact atmospheric conditions desired. The O_2 setpoint range is 1–21%. The O_2 control sensor is located on the blower scroll plate in the chamber unit (*see* **Fig. 2F**). This sensor is a fuel cell that puts out a linear millivolt signal based on O_2 content of the chamber. The O_2 sensor fuel cell depletes over time, depending on required O_2 levels; therefore, the system should be calibrated at least every 6 mo. Two methods are available to calibrate the O_2 system: the preferred method is calibration of the system to the known ambient O_2 value of 20.7%, which checks the life of the sensor; the second method allows the system to be calibrated to an independent reference instrument by entering an offset.

      The Forma 3110 incubator also has a CO_2 thermal conductivity (T/C) sensor. All T/C CO_2 cells are precalibrated at the factory at 37°C, high humidity, and 10% CO_2. Therefore, if a temperature set point of 37°C is entered, the humidity pan filled, and the CO_2 control is to run between 0 and 10% with a T/C CO_2 sensor, the CO_2 set point may be entered immediately. This sensor is not only affected by the quantity of CO_2 present, but also by the air temperature and the water vapor present in the incubator atmosphere. In monitoring the effects of CO_2, air temperature and absolute humidity must be held constant so that any change in thermal conductivity is caused only by a change in CO_2 concentration.

Fig. 2. CO_2 water-jacketed oxygen and eight CO_2-regulated incubator (Model 3110 from ThermoForma, Marietta, OH), which has eight smaller inner doors separating the incubator into eight chambers. **(A)** Temperature display; **(B)** % O_2 display; **(C)** humidity pan; **(D)** water jacket fill port; **(E)** HEPA filter; **(F)** O_2 sensor.

The Forma 3110 incubator has a HEPA filter (*see* **Fig. 2E**) to minimize accumulation of microbial contaminants inside the incubator. During automatic calibration, the CO_2 display is blanked out and HEPA-filtered room air is pumped through the CO_2 sensor. Replacement of the HEPA filter can be set for a specific amount of time, from 1 to 12 mo of actual unit running time. When the allotted time has run out, "REPLACE HEPA" appears in the display and the visual alarm flashes.

For best operation of the incubator, sterilized distilled, demineralized, or deionized water should be used in the humidity pan (*see* **Fig. 2C**). Water

Fig. 3. PROOX model 110 (Reming, Redfield, NY). **(A)** CO_2 controller; **(B)** O_2 controller; **(C)** hypoxia chamber inside a host incubator; **(D)** outer door; **(E)** inner door.

should always be sterilized or treated with a decontaminant that is safe for use with stainless steel and nonvolatile so as not to cause toxicity to the cells. The humidity pan should be filled to within 0.5 in. of the top with sterile, distilled water and placed directly on the incubator floor (*see* **Fig. 2C**) to ensure optimum humidity and temperature response. In addition, the water jacket should be filled with 11.7 gal (42.5 L) of distilled water having a resistance range of 50 K to 1 mΩ/cm using silicone tubing connected directly to the fill port (**Fig. 2D**).

In addition to the standard outer and inner glass door, the 3110 incubator can be equipped with eight smaller inner doors, providing separate inner doors for each shelf of the incubator **(Fig. 2)**, separating the incubator into eight chambers. The advantage of using such an inner door kit is to decrease the gas-mixture disturbance, otherwise inevitable with repeated opening and closing of the incubator door. Incubators should be cleaned and sterilized every 2 to 3 mo.

b. O_2-controlled insert chambers for incubators. The PROOX model 110 from Reming (Redfield, NY) **(Fig. 3)** is a versatile and compact gas oxygen controller for oxygen-sensitive work. The PROOX chamber can be placed inside

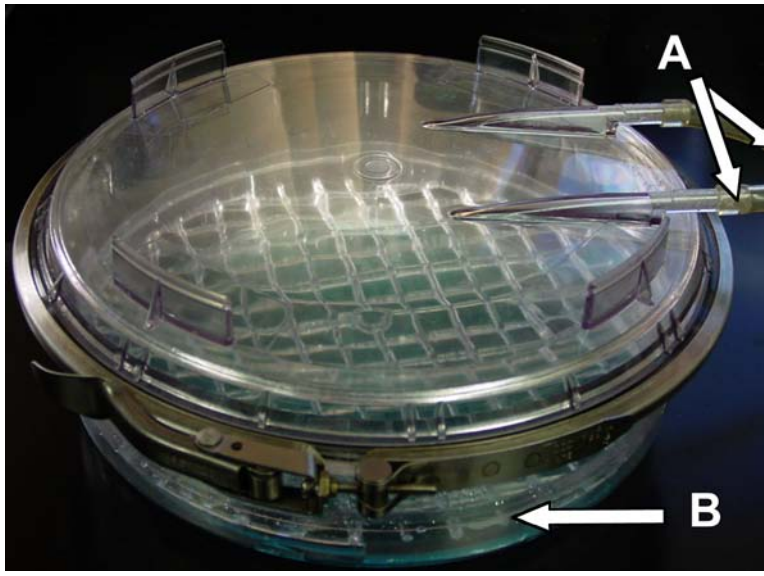

Fig. 4. O_2-controlled chamber (Billups-Rothenberg, Del Mar, CA). The sealed, humidified, modular incubation chamber is flushed with a gas mixture and sealed to obtain hypoxic conditions. **(A)** Inlet and outlet tubes; **(B)** sterile, distilled water.

a standard host incubator, and the O_2 and CO_2 controller panels located outside the host incubator. The host incubator provides the necessary 37°C temperature. An O_2 sensor measures oxygen tension inside the chamber, and if needed infuses nitrogen to displace air and thus lower the oxygen level. Oxygen or oxygen-enriched gas is used to raise oxygen concentrations if hyperoxic conditions are desired. The working range of the incubator is 1–99% O_2. The oxygen controller is connected to the chamber via infusion tubing, and CO_2 levels are maintained by the PROOX chamber via a CO_2 controller connected to a separate CO_2 tank. Control gas must be supplied through a 1/8-in. id hose to the back panel of the PROOX and delivery pressure set at 5–25 psi. The PROOX chamber must be humidified autonomously by placing a stainless steel pan on the chamber floor and filling it periodically with sterile distilled water. The chamber must not be totally sealed, because it can develop a positive pressure, which can damage the sensors. It is important that only limited numbers of culture containers be placed in the chamber to allow for gas circulation needed to establish the hypoxic environment. Repeated access of the chamber can introduce sufficient O_2 to disturb critical experiments.

c. Static (noncontrolled) incubator chambers. Incubator "bubbles" that can be flushed with a gas mixture and sealed, such as those made by Billups-Rothenberg (Del Mar, CA), provide an inexpensive way to maintain a hypoxic cell culture environment **(Fig. 4)** ***(25)***. The chamber has inlet and outlet tubes, allowing con-

nection to a gas cylinder containing the desired oxygen level (0.6–5% O_2), 5% CO_2, and balance nitrogen (95–99.4% N_2). The modular incubation chamber is flushed for 90 s at 10 psi with the gas mixture to displace atmospheric air from the chamber, and then both inlet and outlet tubes are closed simultaneously to avoid underpressure accumulation inside the chamber. The pressure should be approx 5 psi over atmosphere to avoid leaking of O_2 into the chamber. Disadvantages of these chambers are the time required to get in and out of the chamber; the space that they consume, because they are round; and the need for custom gas mixtures. The chamber can be placed in any 37°C incubator. Sterile distilled water should be added in the floor of the chamber (under the plate grating) in order to keep it humidified. It is important that only limited numbers of culture containers be placed in the chamber to allow for gas circulation needed to establish the hypoxic environment and to minimize trapped room air from disturbing the desired O_2 levels in the chamber.

7. Eppendorf P_{O_2} histograph. We used the Eppendorf Histograph (Model KIMOC-6650) to measure in vitro and in vivo oxygen tension. The method (well described in the literature) ***(26)*** utilizes a sterile polarographic electrode consisting of a gold wire contained within a 0.3-mm steel casing. Prior to use, the probe was calibrated by pure, sterile nitrogen bubbled through sterile normal saline. When tumors, murine tissue, or pediatric bone marrow were examined, a maximal track length of 0.5 cm was used.
8. Falcon 96-well microtiter plates (Becton Dickinson, Lincoln Park, NJ).

3. Methods

3.1. Drug Cytotoxicity Assays

1. Harvest and resuspend cells in their appropriate medium, and plate (total volume of 100 µL) in Falcon 96-well microtiter plates at 5000–30,000 viable cells/well (determined by trypan blue dye exclusion), depending on growth characteristics and tumor type; for example, solid tumor cell lines should be seeded at lower cell concentrations (1000–15,000) than leukemia (up to 50,000) due to the appreciable difference in cell size, or the doubling time of a given cell line (slower-growing cells are plated at higher numbers than fast-growing lines).
2. Allow cells to recover overnight. Then add drugs to final concentrations in 50–100 µL of medium, 8–12 wells per drug concentration, with appropriate drug vector added to control wells, and incubate the plates at 37°C for 4–7 d, depending on growth properties ***(27)***. At least three drug concentrations must be tested to be able to calculate lethal drug concentrations. For evaluation of the cytostatic effect of a drug, plates should be incubated for longer periods (14–21 d) and are seeded with fewer cells in order to avoid overgrowth of cells in control wells ***(22)***. However, using the highest number of cells that will not cause overgrowth of controls is advisable to provide the largest dynamic range.
3. To minimize the potential for cytotoxicity, add FDA to the 96-well plates with a final concentration of 10 µg/mL, and incubate for 20 min. One cannot stain more than three to four plates at once with FDA and eosin Y.

4. Add 50 µL of eosin Y (0.5% in normal saline) to quench background fluorescence of the FDA in the medium and in nonviable cells.
5. Measure relative fluorescence by DIMSCAN as described in **Subheading 2., item 4** and express the results as the fractional survival cells compared with control cells.

3.2. Drug Testing Under Reduced Oxygen Conditions

1. For assays in hypoxia, seed cells into plates or flasks and place into one of the three oxygen-controlled systems discussed in **Subheading 2., item 6** incubated at 37°C. Under these conditions using 2% O_2, medium in plates and flasks attains a Po_2 of approx 15 mmHg ***(25)***. This level of oxygen is below the degree of hypoxia found in bone marrow ***(21)*** and in the range of hypoxia found in tumor tissue **(Table 1)**.
3. Dilute drugs from concentrated stock or make freshly in medium preincubated overnight in reduced-oxygen chambers or incubator and in tubes preflushed and capped with reduced-oxygen gas.
4. Rapidly add the drugs to plates to the final concentrations, and quickly place the plates back inside insert hypoxia chambers **(Fig. 3)**, a host incubator, a modular incubator chamber flushed and sealed with the appropriate gas mixture **(Fig. 4)**, or a hypoxia incubator **(Fig. 2)**.
5. If using a modular sealed chamber, after drug addition, reflush with an appropriate O_2 mixture for 90 s, and seal the chamber at approx 5 psi over pressure to reduce atmospheric leaks, incubate at 37°C, and reflush with the appropriate O_2 mixture every other day until assayed.
6. Use the same drug mix tubes to make plates to be studied also in normoxic atmospheres.

4. Notes

1. Cells should be incubated at least overnight in a hypoxia chamber or a hypoxia incubator before any experiment is performed, in order to make cells hypoxic.
2. To minimize the exposure of cells to atmospheric O_2 during drug addition, one should carefully plan experiments and conduct them rapidly.
3. One should be careful not to add more pressure in a hypoxia chamber than is required.
4. All studies determining the effect of hypoxia should be done simultaneously with the experiments performed in normal atmospheric conditions as a control.
5. Incubator chambers must not be fully sealed, because the sensors cannot tolerate positive pressure.
6. Chlorinated tap water should not be used for humidifying an incubator or chambers, because chlorine can deteriorate the stainless steel and may react with drugs. Tap water may also have a high mineral content, which would produce a buildup of scale in the reservoir.

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