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Generation of Oxygen Deficiency in Cell Culture Using a Two-Enzyme System to Evaluate Agents Targeting Hypoxic Tumor Cells

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Abstract

The poor and aberrant vascularization of solid tumors makes them susceptible to localized areas of oxygen deficiency that can be considered sites of tumor vulnerability to pro-drugs that are preferentially activated to cytotoxic species under conditions of low oxygenation. To readily facilitate the selection of agents targeted to oxygen-deficient cells in solid tumors, we have developed a simple and convenient two-enzyme system to generate oxygen deficiency in cell cultures. Glucose oxidase is employed to deplete oxygen from the medium by selectively oxidizing glucose and reducing molecular oxygen to hydrogen peroxide; an excess of catalase is also used to scavenge the peroxide molecules. Rapid and sustained depletion of oxygen occurs in medium or buffer, even in the presence of oxygen at the liquid/air interface. Studies using CHO/AA8 Chinese hamster cells, EMT6 murine mammary carcinoma cells, and U251 human glioma cells indicate that this system generates an oxygen deficiency that produces activation of the hypoxia-targeted prodrug KS119. This method of generating oxygen deficiency in cell culture is inexpensive, does not require cumbersome equipment, permits longer incubation times to be used without the loss of sample volume, and should be adaptable for high-throughput screening in 96-well plates.

INTRODUCTION

Hypoxia is thought to be a driving force behind the genesis of a more aggressive and lethal neoplastic phenotype for a variety of solid tumors (1). Hypoxic regions of tumors not only serve as a focal point for tumor evolution, but the tumors are resistant to radiation therapy because of restricted oxygen levels, and their poor vascularization also limits their accessibility to chemotherapeutic agents (1–3). To study this important property of solid tumor biology, we have developed a relatively simple method to induce hypoxia in cell culture. This system, which we call enzymatically generated oxygen deficiency, uses two readily available enzymes, glucose oxidase and catalase [(4–7) and references therein] to rapidly induce long-term oxygen deficiency in cell cultures without the need for a cumbersome mechanical apparatus or relatively costly equipment. Glucose oxidase rapidly removes oxygen from the cell culture medium or buffer by reducing it with glucose, generating peroxide in the process (4,5).

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DISCLOSURE

Conflict of Interest Statement

The potential anticancer agent KS119 designed and synthesized in Dr. Sartorelli's laboratory has been licensed to Vion Pharmaceuticals, Inc. by Yale University. Dr. Sartorelli currently is a director and Chairman of the Scientific Advisory Board of this company and has common stock in Vion. Three of the authors, R. P. Baumann, P. G. Penketh and K. Shyam, also own stock in Vion.

Peroxide is then removed by the bovine liver enzyme catalase (Fig. 1) (7). Experiments conducted with this system indicate that in the presence of glucose, glucose oxidase rapidly depletes oxygen to near zero in approximately 3 min in buffer or culture medium (Fig. 2).

In these studies we have employed a hypoxic cell-targeted agent developed in this laboratory, 1,2-bis-(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]-carbonyl]hydrazine (KS119), to demonstrate the efficacy of the enzymatically generated oxygen deficiency system. KS119 is a 1,2-bis(sulfonyl)hydrazine prodrug that under-goes enzymatic reductive activation under conditions of oxygen deficiency to generate 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE), which in turn forms hard alkylating species that chloroethylate the O⁶-position of guanine in DNA, ultimately resulting in the formation of a DNA interstrand G-C crosslink (8–11). 90CE has demonstrated anticancer activity (12) and is also the active intermediate formed by Cloretazine, a 1,2-bis(sulfonyl)hydrazine prodrug designed and synthesized in this laboratory (11,13) that has displayed antileukemic activity in relapsed and refractory adult acute myeloid leukemia patients in advanced clinical trials (14).

In the present study, KS119 was found to be preferentially activated under oxygen-deficient conditions generated by enzymatically generated oxygen deficiency in all of the cell lines tested (CHO/AA8, EMT6 and U251). Oxygen deficiency was maintained for at least 6 h under the conditions employed, and after 24 h exposure of cells to KS119, increased cytotoxicity was demonstrated, suggesting that oxygen deficiency was maintained for longer than 6 h. Furthermore, using HPLC analysis and the enzymatically generated oxygen deficiency system, we demonstrated that activation of KS119 is prolonged, with only 20–50% of the parent prodrug disappearing after 24 h of treatment.

MATERIALS AND METHODS

Cell Culture

All cells, CHO/AA8, EMT6 and U251, were maintained under 95% air/5% CO₂ in α -MEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μ g/ml). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

Chemicals

The 1,2-bis(sulfonyl)hydrazine prodrugs and 90CE were synthesized by procedures described previously (10,11). All other drugs and chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO).

Drug Toxicity Studies

Clonogenic survival assays were performed essentially as described previously (15). Cells were seeded into plastic 25-cm² tissue culture flasks at $5\text{--}8 \times 10^5$ cells per flask, grown for 1–2 days, and then pulsed with KS119 dissolved in DMSO in a total volume of 10 ml of medium for 2 to 24 h at 37°C. For some of the 2-h experiments with enzymatically generated oxygen deficiency, as specified in the figure legend, cells were pretreated for 1 h without drug in the presence of 2 U/ml of glucose oxidase (Sigma G6641), 120 U/ml of catalase (Sigma C1345), and 10 mM of added glucose (final glucose concentration 14.5–15.0 mM). After pretreatment, KS119 was added, and treatment continued for an additional 2 h. For time-course and longer drug exposure experiments (2, 4, 6 and 24 h), no pretreatment was performed, and enzymes, glucose and KS119 were added simultaneously. Flasks were flushed with nitrogen for 10 s and the caps were screwed on tight. This facilitates oxygen depletion of the medium by glucose oxidase by removing residual oxygen-containing air and preventing the entry of additional air. After treatment, monolayers were rinsed, and cells were detached by trypsinization, suspended in culture medium, and counted; sequential cell dilutions were plated in duplicate into six-well

plates at a density of 1×10^2 , 1×10^3 or 1×10^4 cells per well. Seven to 10 days later, colonies were fixed, stained with crystal violet in 80% methanol, and counted. All analyses were corrected for plating efficiency in the presence of vehicle (DMSO) at concentrations equivalent to those used for the exposure to KS119. As expected, cell plating efficiencies decreased under enzymatically generated oxygen deficiency for 24 h, but only by 20% or less; only minor variations were seen with a 3-h enzymatically generated oxygen deficiency treatment (Fig. 3A and B).

For oxygen depletion experiments conducted by conventional nitrogen flush, cells were seeded in 25-cm² plastic flasks at 2.5×10^5 cells per flask and grown for 3 days in a humidified atmosphere of 95% air/5% CO₂. Oxygen deficiency was established by gassing the cultures with a humidified mixture of 95% N₂/5% CO₂ (containing ≤ 10 ppm O₂; AirGas) at 37°C for 2 h through a rubber septum fitted with 13-gauge (inflow) and 18-gauge (outflow) needles. The cultures were then incubated with KS119 at various concentrations for 2 h. Cells under aerobic conditions were treated identically but were gassed with a humidified mixture of 95% air/5% CO₂. Cells were then washed, harvested by trypsinization, and assayed for survival using a clonogenic assay described previously (8,16).

Oxygen Depletion by Enzymatically Generated Oxygen Deficiency in Model Systems

The measurement of oxygen depletion and the duration of depletion generated by the enzymatically generated oxygen deficiency system was performed in a 2-ml 0.8-cm-diameter well exposed to air using an oxygraph (YSI model 5300 biological oxygen monitor). In these experiments, the solution was stirred in a manner that represented the most unfavorable case, wherein there is equilibration of the O₂ concentration in the aqueous phase and the entry of O₂ is driven by the full O₂ concentration gradient across the meniscus. Early experiments indicated that the enzymatically generated oxygen deficiency system could maintain the aqueous phase in a highly O₂-depleted state under such conditions (note that the oxygraph requires a stirred solution to function). While an air-exposed stirred solution is the least favorable condition to maintain oxygen depletion since it results in the highest rate of O₂ entry and a strong gradient over a short spatial range, stirring ensures a uniform oxygen concentration throughout the solution. Initial experiments indicated that the enzymatically generated oxygen deficiency system was capable of sustaining the oxygen-depleted condition for several hours under this highly unfavorable condition of stirring and unlimited oxygen supply. The use of unstirred solutions over cell monolayers in sealed flasks where oxygen is limited results in longer-lasting oxygen deficiency in the region of the cells, and once oxygen is completely depleted in a sealed cell culture flask, oxygen deficiency can be maintained indefinitely. Calculations of oxygen depletion with stirred solutions are useful since they represent the worst-case scenario. If stirring does not abolish the oxygen depletion, transient agitation during the addition of experimental components will not perturb the system. In these mixtures (stirred and air-exposed), the O₂ concentration is decreased to a low steady-state level for an extended period and the O₂ concentration returns to the normal air-saturated level once the glucose nears exhaustion.

Approximate Calculation of the Steady-State O₂ Concentration and Duration of Oxygen Depletion in Stirred Solutions

At steady state the rate of O₂ entry equals the rate of O₂ consumption. Entry rate = constant $\times$ surface area $\times$ concentration gradient. The concentration gradient is expressed as a fraction: $(K_S - [O_{2ss}])/K_S$, where K_S is the oxygen concentration in air-saturated buffer at 37°C and pH 7.4 (217 μ M). $[O_{2ss}]$ is the steady-state O₂ concentration. Therefore, Entry = $K_E \times SA \times (K_S - [O_{2ss}])/K_S$.

When substrates are far below their K_m values, the rates of the reaction are approximately proportional to their concentrations. The published K_m of glucose oxidase is 110 mM for glucose and 200 μM for O_2 . Since we are using 14.5 mM glucose and are seeking to achieve O_2 concentrations that are less than 2% of the air-saturated level (i.e., around 4–5 μM), both substrates are present at much lower levels than their K_m values. Therefore, the rate of O_2 consumption at steady state will be expected to be approximately proportional to the concentration of both substrates, the enzyme and the medium volume.

$$\text{Consumption} = [O_2ss][\text{Glucose}][\text{Enz}][\text{Vol}]K_C.$$

Since at steady state consumption is equal to entry, $K_E \times SA \times (K_S - [O_2ss])/K_S = [O_2ss][\text{Glucose}][\text{Enz}][\text{Vol}]K_C$, we can combine the two constants K_E , the entry constant, and K_C , the consumption constant such that $K_C/K_E = K$.

When solved for the steady-state O_2 concentration, the equation then becomes

$$[O_2ss] = 217 \times 10^{-6} / ((k \times [\text{glucose}] [\text{Enz}] (\text{Vol}/SA)) + 1).$$

Experiments measuring the steady-state O_2 concentration using an oxygraph over a range of glucose concentrations (2–20 mM) and glucose oxidase concentrations (0.5–8 U/ml) showed that while this equation matches the experimental data with respect to changes in enzyme activity, the $[O_2ss]$ was much less dependent on the glucose concentration than the above equation predicts. Hence the equation was modified empirically to match the relationships observed experimentally:

$$\begin{aligned} \text{Steady-state } [O_2] \text{ } \mu M \\ = 217 \times 10^{-6} / ((2.52 \times [\text{Glucose}]^{1/3} \times [\text{Enz}] \times (\text{Vol}/SA)) + 1), \end{aligned}$$

where $[\text{Glucose}]$ is the glucose concentration in mM, $[\text{Enz}]$ is the enzyme concentration in U/ml, Vol is the mixture volume in ml, and SA is the surface area exposed to the air in cm^2 .

The low dependence on glucose concentration is advantageous because there will be a relatively small change in the steady-state O_2 concentration as the glucose in the medium is consumed. The duration of oxygen deficiency (hypoxia) is a simple calculation if we consider that at high levels of O_2 depletion the concentration gradient will be relatively constant at its maximum level. Therefore, the O_2 entry will be essentially proportional to the surface area, and the duration of oxygen depletion will be the time taken to consume the available glucose, assuming that sufficient glucose oxidase activity is present to keep the oxygen concentration low until most of the glucose is exhausted. This results in an equation of the form

$$\text{Duration of hypoxia} = K_D [\text{Glucose}] \text{Vol}/SA,$$

where K_D is the duration constant at 37°C for a stirred solution, which is measured experimentally as 4.7.

The situation is slightly complicated because it takes a non-trivial amount of glucose (0.43 mM, measured experimentally) to establish the hypoxia initially, so the final equation is

$$\begin{aligned} &\text{Duration of hypoxia in minutes} \\ &= 4.7 \times \text{Vol/SA} \times ([\text{Glucose}] - 0.43). \end{aligned}$$

These relationships provide a guide to the minimum extent and duration of oxygen deficiency.

HPLC Determination of the Disappearance of KS119 in Cell Cultures under Conditions of Oxygenation and Oxygen Depletion

Experimental samples containing KS119 were mixed with an equal volume of acetonitrile and allowed to stand at room temperature for 15 min to allow precipitation of most of the protein, then centrifuged at 10,000g for 5 min. The supernatant was then analyzed by HPLC using a 5- μ m 220 $\times$ 4.6-mm C-18 reverse-phase column (RP-18, Applied Bio-systems); elution was accomplished with 34.5% acetonitrile in buffer (0.03 M KH_2PO_4 /1.0 mM NaN_3 , pH 5.4) for 5 min, followed by a 34.5 to 75.0% acetonitrile linear gradient in buffer, at a flow rate of 0.6 ml/min. Absorbance was monitored at 280 nm using a 168 UV/Vis detector (Beckman Coulter, Fullerton, CA). KS119 eluted as two isomeric conformers in a slightly split single peak at 35 min.

RESULTS

Oxygen Depletion by Glucose Oxidase in Model Systems

In the presence of molecular oxygen, glucose oxidase oxidizes glucose to produce gluconic acid and peroxide. Catalase then removes peroxide, a potentially toxic product, by converting it to water and oxygen, resulting in the consumption of two molecules of glucose per oxygen molecule (Fig. 1). This uncomplicated two-enzyme system, which results in the rapid depletion of oxygen from the liquid phase, is the basic catalytic foundation of enzymatically generated oxygen deficiency. As illustrated in Fig. 2, depletion of oxygen in cell culture medium or buffer, as measured with an oxygraph, occurs rapidly with small amounts of enzyme (Fig. 4A). Thus less than 1 U of glucose oxidase per ml was able to quickly deplete oxygen to near zero in an unsealed reaction mixture. The level of depletion of oxygen by glucose oxidase was dependent on the glucose concentration (Fig. 4B). In studies duplicating the cell culture conditions employed, the oxygen level was found to be depleted to under 1% in 1 min and to near zero within 3 min of enzyme addition. In the absence of stirring, using our standard surface area to volume ratios (0.25), or in sealed systems, using 2 U/ml of glucose oxidase and 120 U/ml of catalase, the rate of oxygen consumption greatly exceeded the rate of oxygen entry. As a result, the minimum amount of glucose required to deplete liquid dissolved oxygen is only slightly greater than twice the molar concentration of dissolved oxygen in the sample. At pH 7.4 and 37°C, the concentration of dissolved O_2 at air saturation is approximately 200 μM , so that approximately 450 μM glucose is required to deplete all of the dissolved O_2 in the liquid. Under conditions of constant stirring while being exposed to the atmosphere in the oxygraph, a minimum of 2 mM glucose was required to establish a steady-state concentration of oxygen, with >96% depletion of O_2 . These experimental studies demonstrate that oxygen depletion in both unstirred and stirred systems is feasible. These findings prompted us to use this method to test the depletion of oxygen in cell culture using the hypoxia-activated agent KS119.

KS119 Activation Studies

The ability of rationally designed prodrugs to undergo selective activation in the hypoxic regions of solid tumors is an area of intensive investigation in our laboratory (8–11). To determine whether the enzymatically generated oxygen deficiency method can be employed to evaluate agents requiring activation by hypoxic tumor cells, as currently measured with a nitrogen flush hypoxia system (8), we evaluated the hypoxia-activated agent KS119 using this

system. As shown in Fig. 5A–C, when tested in cells of three different cell lines, CHO/AA8 Chinese hamster cells, EMT6 murine mammary carcinoma cells and U251 human glioma cells, preferential activation of KS119 was demonstrated under conditions of oxygen depletion generated by the enzymatically generated oxygen deficiency system. Cells were pretreated for 1 h with 2 U/ml of glucose oxidase and 120 U/ml of catalase in 10 ml of medium, with added glucose at a final concentration 14.5–15.0 mM. After 1 h, KS119 was added and cells were incubated for an additional 2 h before plating. As anticipated, minimal cell toxicity was seen under oxygenated conditions, since redox cycling of enzymatically activated KS119 under oxygenated conditions prevents reductive activation to a cytotoxic species (8,9). In contrast, using the enzymatically generated oxygen deficiency system, cell survival was reduced by 1.5 to 3.0 logs using KS119 at a concentration of 40 μ M (Fig. 5).

Cell Culture Parameters Influencing Enzymatically Generated Oxygen Depletion

The enzymatically generated oxygen deficiency system results in a rapid depletion of oxygen in the culture medium, thereby generating an oxygen-deficient liquid barrier from the air in the culture flask that contains residual oxygen. This method does not rely on the cellular depletion of oxygen in the medium since this is accomplished directly by the glucose oxidase; therefore, oxygen depletion occurs within minutes. Certain cell culture variables have the potential to influence the establishment and maintenance of oxygen deficiency. These include the volume of medium employed, the cell density and the starting glucose level. Experiments were routinely conducted with 10 ml of medium in 25-cm² flasks since this volume of medium was empirically determined to be optimum for this system.

Experiments were also performed to evaluate the influence of cellular density on cytotoxicity in the enzymatically generated oxygen deficiency system. As anticipated, the highest cell densities produced the greatest cell toxicity (Fig. 6). These results were expected, since KS119 requires activation by cellular reductase enzymes to generate a cytotoxic species. Therefore, the greater the number of cells, the more KS119 activation, the greater the concentration of cytotoxic products, and therefore the greater the cell killing.

To demonstrate that the enzymatically generated oxygen deficiency method is functional for other agents that can be preferentially activated under conditions of oxygen depletion, mitomycin C was tested in EMT6 cells under normal oxygenation and oxygen depletion (Fig. 7). As anticipated, mitomycin C displayed considerably greater cytotoxicity under enzymatically generated oxygen deficiency than under conditions of oxygenation, demonstrating that the differential cytotoxicity obtained with this method is not unique to KS119. Studies performed with drugs generating a toxic lesion similar to that of KS119 (90CE and Cloretazine) but not requiring oxygen-depleted conditions for activation showed equal toxicity under oxygen depletion with enzymatically generated oxygen deficiency and oxygenation (results not shown).

To compare the results from the enzymatically generated oxygen deficiency system to the more traditional nitrogen flush method of generating oxygen depletion in cell culture, experiments were conducted in cells of all three cell lines using nitrogen gas to deplete the oxygen level. As shown in Fig. 8 by comparison to Fig. 5, the enzymatically generated oxygen deficiency system is comparable to the nitrogen purge method in generating oxygen-depleted killing with KS119 in all three of the cell lines used in the present studies.

The early availability of glucose is critical to the enzymatically generated oxygen deficiency assay, since glucose oxidase cannot remove oxygen from the medium without this substrate. In addition, cells use glucose and therefore can be expected over time to consume glucose and to deplete the level of this substrate on their own. In our experiments, we normally used a starting glucose level of approximately 15.0 mM; the medium employed, α -MEM, normally

contains approximately 4.5–5.0 mM glucose, and this was augmented to 14.5–15.0 mM prior to the addition of enzymes.

Glucose levels were also measured directly with an oxygraph over time using glucose oxidase. These studies indicated that oxygen depletion in liquids can be maintained under unsealed conditions in the presence of abundant air with glucose levels of ≥ 2 mM, while under sealed conditions where oxygen is limited, only approximately 400 μ M glucose is required to maintain the oxygen depletion (Table 1). Measurement of glucose levels in spent medium after incubation with cells in sealed cell culture flasks indicated that sufficient glucose was available to maintain oxygen depletion for over 6 h (Table 2). Flasks were pulsed with nitrogen for 10 s and then the tops were screwed on tight, except where indicated; therefore, little if any oxygen remained in the residual air trapped in the flask after 6 h of enzymatically generated oxygen deficiency and no additional atmospheric oxygen could enter the flask. Thus it is likely that oxygen depletion is maintained in the flasks for considerably longer than 6 h under these culture conditions. This was tested by measuring glucose levels under enzymatically generated oxygen deficiency in medium from CHO/AA8 cell cultures at 3-h intervals over a 24-h period (Fig. 9). Glucose levels stayed within the 0.4 mM threshold needed to maintain oxygen deficiency for 21 h. Since glucose is also likely to be important in drug reduction, this experiment indicates that drug activation by glucose-dependent mechanisms should still occur even after prolonged treatment under enzymatically generated oxygen deficiency. Where necessary to ensure maintenance of long-term oxygen depletion and drug activation, extra glucose could be added above the levels employed in our experiments or a medium could be used for incubation that contains higher starting levels of glucose, for example Dulbecco's modified Eagle's medium (25 mM glucose). Furthermore, as shown in Fig. 10A–C and Table 3, when incubation times were varied for KS119 exposure under enzymatically generated oxygen deficiency or normal oxygenation from 2 to 24 h, progressive cytotoxicity was observed. These experiments support the interpretation that drug activation is prolonged, an observation also supported by HPLC studies.

Thus one of the important features of the enzymatically generated oxygen deficiency assay is the potential for easily extended incubation times under oxygen depletion. This feature is particularly useful for agents such as KS119 that are not rapidly metabolized or activated by cells. HPLC experiments designed to measure residual KS119 levels after incubation in cell culture indicated that only 20 to 50% of the initial concentration of KS119 prodrug is metabolized and/or activated after 24 h of incubation with oxygen-depleted cells (Table 4). These studies do not distinguish between metabolism of KS119 to innocuous products and prodrug activation of KS119 to the reactive chloroethylating species, since all of these products are not detectable by HPLC analysis under the conditions employed to measure parental KS119.

DISCUSSION

Hypoxic regions of tumors, while important in the development of aggressive cancers, are also a distinguishing feature of solid tumors that can be directly targeted by chemotherapeutic prodrugs designed to be preferentially activated within cells by reductive enzymes under conditions of oxygen deficiency. KS119 is a prodrug synthesized and evaluated in this laboratory as an agent activated preferentially by hypoxic cells (8). In this paper, we describe a versatile, convenient and rapid method for generating long-term oxygen-deficient conditions in cell culture using an enzymatic mechanism, which we have termed enzymatically generated oxygen deficiency. This method does not require cumbersome or expensive equipment, can be used for large experiments with multiple samples, and can induce oxygen deficiency for at least 6 h. Our studies have shown that glucose oxidase in the presence of glucose rapidly induces oxygen deficiency within minutes in culture medium or buffer while generating peroxide in

the process (Fig. 1). A vast excess of catalase added to the reaction rapidly removes peroxide from the medium to prevent any cytotoxicity due to the accumulation of peroxide, but there is a possibility that low levels of hydrogen peroxide present in the initial 1–3 min before oxygen is completely depleted in the medium might alter cellular properties and modify results in some experimental situations in which it would be desirable to employ enzymatically generated oxygen deficiency. This potential caveat should be considered by investigators interested in employing enzymatically generated oxygen deficiency for cell lines that are particularly susceptible to the effects of low levels of hydrogen peroxide. Catalase is a unique enzyme, one of the fastest known, not saturable by substrate and with kinetics that approximate first order (20). Our calculations indicate that the half life of hydrogen peroxide under enzymatically generated oxygen deficiency is 12 s, with a maximum transient concentration of 30 μM at about 1 min, which decays to zero within the 3 min (or less) it takes glucose oxidase to completely deplete oxygen from the medium (Baumann *et al.*, unpublished observation). After oxygen removal from the medium, the only generation of peroxide that can occur due to glucose oxidase is at the liquid/air interface. Any per-oxide generated at this site would need to diffuse through a gauntlet of a large excess of catalase to reach the attached cells beneath the medium.

Clonogenic assays conducted with mouse EMT6 cells, hamster CHO/AA8 cells and human glioma U251 cells demonstrated that the enzymatically generated oxygen deficiency system produces preferential KS119 cytotoxicity comparable to that generated by the nitrogen flush technique using 95% N_2 /5% CO_2 , which results in medium containing ≤ 10 ppm of oxygen. These findings suggest that the enzymatically generated oxygen deficiency system results in a degree of oxygen depletion similar to the more established nitrogen flush system. This level of oxygen depletion may raise concern by some investigators that this degree of oxygen depletion may not occur in solid tumors *in situ*. However, there is evidence produced by Vaupel and colleagues that this degree of oxygen depletion does indeed occur in solid tumors. These authors state in two important reviews (18,19) that “in solid tumors oxygen delivery to respiring neoplastic and stromal cells is frequently reduced or even abolished by a deteriorating diffusion geometry, severe structural abnormalities of tumor microvessels and distorted microcirculation. In addition, anemia and the formation of methemoglobin or carboxyhemoglobin reduces the blood’s capacity to transport O_2 . As a result, areas with very low (down to zero) oxygen partial pressures exist in solid tumors, occurring acutely or chronically. These microregions of very low or zero O_2 partial pressure are heterogeneously distributed within the tumor mass.”

HPLC studies revealed that the activation of the KS119 prodrug occurs relatively slowly, is prolonged, and is not complete even after 24 h of incubation under oxygen depletion. Depending on the cell line employed, only 20 to 50% of the initial KS119 disappeared during a 24-h incubation period. This was surprising considering the relatively great cytotoxicity of this compound under conditions of oxygen depletion, suggesting that it is highly targeted to cells; this property is probably attributable to the requirement that KS119 be directly converted to the active form by cellular enzymes, which produce only net activation of KS119 under oxygen deficiency combined with the short half-life of 30 s (allowing little time for diffusional escape) of its generated reactive cytotoxin, 90CE, which leads to the alkylation of the O^6 position of guanine in DNA. The observation that cell killing increased at 24 h over that occurring at 6 h suggests that oxygen deficiency was maintained in sealed flasks for this period, even in the presence of low levels of glucose. This phenomenon occurred because after 6 h oxygen had been completely depleted from the air within the flask and could not be replenished from the outside. The unique features of the enzymatically generated oxygen deficiency method may make it particularly suitable for a rapid microtiter-based assay for agents that undergo activation under conditions of oxygen deprivation.

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Net Reaction:

FIG. 1.
Schematic diagram depicting the reactions occurring in enzymatically generated oxygen deficiency (EGOD).

FIG. 2.

Schematic diagram depicting the generation of oxygen deficiency by the two-enzyme system of glucose oxidase and catalase under the conditions employed. The oxygen concentration is rapidly (<2 min) reduced to a low steady-state level. This low steady state is maintained for 3 h under stirred unsealed conditions using 10 mM glucose. When glucose is nearly depleted, the O_2 concentration returns to the normal saturated value over 30 min.

FIG. 3. Clonogenic experiments using CHO/AA8, U251 and EMT6 cells to measure plating efficiency either under conditions of oxygen depletion or under normal oxygenation. Panel A: 3 h; panel B: 24 h. Values are means $\pm$ SEM from at least three independent experimental determinations.

FIG. 4.

The effects of various conditions on the maximum level of oxygen depletion attained in a 2-ml chamber with a surface area of 0.5 cm² as measured polarographically using a Yellow Springs Instrument Company oxygraph. Panel A: The effects of increasing the level of glucose oxidase (GO)/catalase activity at a constant 10 mM glucose concentration in a stirred 100 mM potassium phosphate buffer, pH 7.4 (catalase present at ~60-fold the glucose oxidase activity in U/ml). Panel B: The effects of varying the concentration of glucose in the presence of 0.25 U/ml of GO and 2 U/ml of GO with stirred air-exposed solutions, and with 2 U/ml of GO under stirred and sealed conditions.

FIG. 5.

Survival of cells treated with KS119 under either conditions of oxygen depletion produced by enzymatically generated oxygen deficiency or under oxygenation. CHO/AA8 (panel A), U251 (panel B) and EMT6 (panel C) cells were treated with varying concentrations of KS119 under conditions of oxygen depletion (▲) and oxygenation (■). Oxygen-depleted samples were pretreated for 1 h by enzymatically generated oxygen deficiency methodology, 2 U/ml of glucose oxidase, 120 U/ml of catalase and 14.5 mM glucose, then for another 2 h in the presence of KS119 before cells were harvested and plated. Points are the means $\pm$ SEM at least three independent determinations.

FIG. 6. The influence of cell density on cytotoxicity. CHO/AA8 cells were pretreated for 1 h by enzymatically generated oxygen deficiency, then treated with 10 μ M KS119 for 2 h under oxygen depletion. Means $\pm$ SEM, $n \geq 3$.

FIG. 7.

Survival of EMT6 cells treated with varying concentrations of Mitomycin C under conditions of oxygen depletion (▲) and normal oxygenation (■). Oxygen-depleted samples were pretreated for 1 h by enzymatically generated oxygen deficiency methodology, 2 U/ml of glucose oxidase, 120 U/ml of catalase and 14.5 mM glucose, then for another 2 h in the presence of Mitomycin C before harvesting and plating cells. Points are the means $\pm$ SEM for at least three independent experimental determinations.

FIG. 8.

The effects of varying concentrations of KS119 on cell survival under conditions of oxygen depletion produced by nitrogen flushing or under normal oxygenation. CHO/AA8 cells (panel A), U251 cells (panel B) and EMT6 cells (panel C) were treated with varying concentrations of KS119 under conditions of oxygen depletion ($\blacktriangle$) and oxygenation ($\blacksquare$). Oxygen-depleted samples were pretreated for 1 h with continuous nitrogen flow, then for another 2 h in the presence of KS119 before harvesting and plating.

FIG. 9.

Residual glucose concentration in cell medium of CHO/AA8 cell cultures as a function of time. Five $\times 10^5$ cells were plated in each flask, and 24 h later fresh medium with additional glucose was added with incubation maintained under conditions of oxygen depletion by enzymatically generated oxygen deficiency for 24 h. Every 3 h, samples were boiled to inactivate glucose oxidase and catalase, and glucose levels were then measured. Points are means $\pm$ SEM. The 0.4 mM threshold for oxygen depletion is indicated by the dashed line.

FIG. 10.

Survival of cells treated with 10 μM KS119 for varying times under conditions of oxygen depletion using enzymatically generated oxygen deficiency or under normal oxygenation. CHO/AA8 cells (panel A), U251 cells (panel B) and EMT6 cells (panel C) were treated with KS119 under conditions of oxygen depletion or normal oxygenation for 2 to 24 h. Points are means $\pm$ SEM, $n \geq 3$. Oxygen depletion was generated by the enzymatically generated oxygen deficiency system after the addition of 2 U/ml of glucose oxidase, 120 U/ml of catalase and 14.5–15.0 mM glucose.

TABLE 1

The Dependence of Oxygen Depletion on Glucose Levels in Model Systems

Unsealed ^a		Sealed	
Glucose (mM)	Oxygen (%) ^b	Glucose (mM)	Oxygen (%) ^b
1	(3.8 ± 0.35) ^c	0.05	16.7 ± 0.8
2	1.1 ± 0.1	0.1	14.3 ± 0.4
4	0.8 ± 0.05	0.25	6.8 ± 0.2
10	0.7 ± 0.06	0.5	0 ± 0
20	0.55 ± 0.04	1	0 ± 0

^a All reactions were carried out at 37°C in 100 mM potassium phosphate buffer, pH 7.4, containing 2 U/ml of glucose oxidase and 120 U/ml of catalase. In unsealed experiments the reported oxygen concentration represents the lowest steady-state concentration achieved. In the sealed experiments the reported oxygen concentration represents a stable O₂ concentration achieved at either glucose or oxygen exhaustion.

^b Oxygen levels were determined using an oxygraph and are given as means ± SEM (*n* ≥ 3).

^c Value in parentheses was transient and was not sustained.

TABLE 2
Residual Glucose Concentration in Expended Cell Medium as a Function of Time

Time (h) ^a	CHO/AA8 ^b		U251 ^b		EMT6 ^b	
	Oxygen depleted	Oxygenated	Oxygen depleted	Oxygenated	Oxygen depleted	Oxygenated
0	15.3 ± 0.3	5.2 ± 0.006	15.3 ± 0.3	5.2 ± 0.006	15.3 ± 0.3	5.2 ± 0.006
6	9.0 ± 0.2	5.4 ± 0.2	9.0 ± 0.6	4.8 ± 0.1	9.4 ± 0.7	4.4 ± 0.1
24	0.03 ± 0.006	0.7 ± 0.07	0.02 ± 0.008	2.8 ± 0.4	0.04 ± 0.003	0.1 ± 0.04

^aTime after the addition of glucose oxidase (2 U/ml) and catalase (120 U/ml); no pretreatment was performed.

^bSamples of medium from individual flasks containing attached cells were boiled to inactivate glucose oxidase and catalase, and glucose was measured using a glucose oxidase standardization assay. Values are given in mM ± SEM (*n* = 3).

TABLE 3

Differential Cytotoxicity Produced by KS119 under Oxygen-Depleted and Oxygenated Treatment

Time (h) ^a	CHO/AA8 ^b	U251 ^b	EMT6 ^b
2	11.9	1.1	10.4
4	23.7	4.8	44.7
6	52.2	6.8	67.4
24	53.2	21.5	35.3

^aTime after the addition of KS119 and glucose oxidase; no pretreatment was performed. Values were derived from experiments shown in Fig. 10.

^bValues are given as the ratio of the cell killing under conditions of oxygen depletion divided by the cell killing produced under normal oxygenation at the same incubation times.

TABLE 4

HPLC Determination of KS119 Metabolism by Different Cell Lines

Cell line ^a	Disappearance of KS119 ^b	
	Oxygenated	Oxygen depleted
CHO/AA8	-2.3 ± 1.2	15 ± 1
U251	-1.7 ± 0.7	7.7 ± 1.8
EMT6	11 ± 2	37 ± 6

^a Cells (0.5×10^6 per flask) were treated with KS119 under conditions of oxygen depletion using the EGOD system for 24 h or under oxygenation. Oxygen depletion was generated by the EGOD system after the addition of 2 U/ml of glucose oxidase, 120 U/ml of catalase and 14.5–15.0 mM glucose.

^b Samples of medium from individual flasks were mixed 1:1 with acetonitrile and subjected to HPLC analysis to determine the KS119 content. Values are the percentage of the KS119 that disappeared ± SEM ($n = 3$) compared to controls assayed in the absence of cells. The half-life for KS119 in medium at 37°C has been determined to be approximately 4 days in the absence of cells, or approximately 15.7% disappearance over 24 h under oxygenation or 16.5% under oxygen deficiency.