1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine: An anticancer agent targeting hypoxic cells

Helen A. Seow*, Philip G. Penketh*†, Krishnamurthy Shyam*†, Sara Rockwell*‡§, and Alan C. Sartorelli*†¶

Departments of *Pharmacology and ‡Therapeutic Radiology and §Developmental Therapeutics Program, Yale Cancer Center, Yale University School of Medicine, New Haven, CT 06520

Edited by Anthony Cerami, Kenneth S. Warren Institute, Kitchawan, NY, and approved May 16, 2005 (received for review December 3, 2004)

To target malignant cells residing in hypoxic regions of solid tumors, we have designed and synthesized prodrugs generating the cytotoxic alkylating species 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE) after bioreductive activation. We postulate that one of these agents, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119), requires enzymatic nitro reduction to produce 90CE, whereas another agent, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(4-nitrobenzyloxy)carbonyl]hydrazine (PNBC), can also be activated by nucleophilic attack by thiols such as glutathione (GSH)/GST. We demonstrated that these agents selectively kill hypoxic EMT6 mouse mammary carcinoma and CHO cells. In hypoxia, 50 μM KS119 produced 5 logs of kill of EMT6 cells without discernable cytotoxicity in air; similar effects were observed with CHO cells. PNBC was less efficacious against hypoxic tumor cells and also had some toxicity to aerobic cells, presumably because of GST/thiol activation, making PNBC less interesting as a selective hypoxic-cell cytotoxin. BALB/c mice with established EMT6 solid tumors were used to demonstrate that KS119 could reach and kill hypoxic cells in solid tumors. To gain information on bioreductive enzymes involved in the activation of KS119, cytotoxicity was measured in CHO cell lines overexpressing NADH:cytochrome *b*5 reductase (NBR), NADPH:cytochrome P450 reductase (NPR), or NAD(P)H:quinone oxidoreductase 1 (NQO1). Increased cytotoxicity occurred in cells overexpressing NBR and NPR, whereas overexpressed NQO1 had no effect. These findings were supported by enzymatic studies using purified NPR and xanthine oxidase to activate KS119. KS119 has significant potential as a hypoxia-selective tumor-cell cytotoxin and is unlikely to cause major toxicity to well oxygenated normal tissues.

alkylation | carbamylation | cloretazine | VNP40101M

Successful treatment of solid tumors is limited by populations of viable malignant cells in hypoxic regions of the tumor. Hypoxia is known to increase metastatic potential through multiple mechanisms, such as the induction of angiogenesis, the promotion of tissue remodeling, and the selection of cell populations resistant to apoptosis (for a review, see ref. 1). In hypoxic areas, low oxygen tension also minimizes the cytotoxic effects of ionizing radiation, and cells in these areas may survive x-irradiation and repopulate the tumor (2). Also, because hypoxic regions are often distal to the tumor vasculature, many cancer chemotherapeutic agents cannot reach these areas at sufficiently cytotoxic concentrations (3). Furthermore, hypoxic tumor cells can be either quiescent or slowly progressing through the cell cycle; therefore, cell-cycle-active drugs may be ineffective. However, we have considered hypoxic areas in tumors to be sites of vulnerability, in that their environment promotes reductive processes, creating an exploitable difference between cells in normal tissues, which are generally well oxygenated, and hypoxic neoplastic cells (4).

Various therapies using prodrugs that require bioreduction to generate active antineoplastic agents have been developed to target the hypoxic regions in solid tumors. Thus, agents such as mitomycin C, tirapazamine, and 5-aziridinyl-3-hydroxymethyl-1-methyl-2-[1H-indole-4,7-dione]prop-2-en-1-ol (E09) use the bioreductive environment of hypoxic tumor cells for preferential generation of a cytodestructive species (5–10). We have synthesized a series of sulfonylhydrazine prodrugs that are capable of generating 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE), an alkylating species that targets the O^6-position of guanine residues in DNA after reductive activation (11, 12). Two of the agents of this class, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119; ref. 13) and 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(4-nitrobenzyloxy)carbonyl]hydrazine (PNBC; refs. 13 and 14) use a nitro group as the trigger to induce the formation of the cytodestructive 90CE form. The chemical structures of these agents are shown in Fig. 1.

Activation of nitro aromatic compounds is initiated by means of the addition of one electron by a bioreductive enzyme to form a nitro radical anion. Under aerobic conditions, the nitro radical anion is rapidly back-oxidized by O_2 to regenerate the inactive prodrug form and $O_2^{\cdot-}$, resulting in an undetectably low steady-state concentration of nitro radical anions (15). However, under hypoxic conditions, the nitro radical anion probably undergoes disproportionation and further reduction through the addition of successive electrons to produce nitroso, hydroxylamino, and amino species, as demonstrated by *p*-substituted nitrobenzenoids (16). Conversion of the electron-withdrawing nitro group to either the electron-releasing hydroxylamino or amino functionality results in the activation of the drug to produce the desired cytotoxic species (17). This activation mechanism is shown in Fig. 2*A*. An alternate mechanism for the activation of PNBC, but not KS119, is the thiolytic activation catalyzed by GST, as shown in Fig. 2*B*.

The anticancer activity of the chloroethylating species 90CE has been documented in studies evaluating the sulfonylhydrazine prodrug 1,2-bis(methylsulfonyl)-1-[(2-chloroethyl)-2-(methylamino)carbonyl]hydrazine (Cloretazine, VNP40101M, 101M), which is activated by base catalyzed decomposition (12, 18, 19). Cloretazine has exhibited a broad spectrum of antitumor activity

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: 90CE, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine; KS119, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine; NBR, NADH:cytochrome *b*5 reductase; NPR, NADPH:cytochrome P450 reductase; NQO1, NAD(P)H:quinone oxidoreductase 1; PNBC, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(4-nitrobenzyloxy)carbonyl]hydrazine; XO, xanthine oxidase; GSH, glutathione.

†The agent described in this article has been licensed by Yale University to Vion Pharmaceuticals, Inc. A.C.S. is a Director and Chairman of the Scientific Advisory Board of Vion Pharmaceuticals. Vion has provided A.C.S.'s laboratory with a relatively small gift; none of these monies were used to support the research described here. Additionally, A.C.S., P.G.P., and K.S. are stockholders in Vion Pharmaceuticals.

¶To whom correspondence should be addressed at: Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208066, New Haven, CT 06520. E-mail: alan.sartorelli@yale.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0409013102

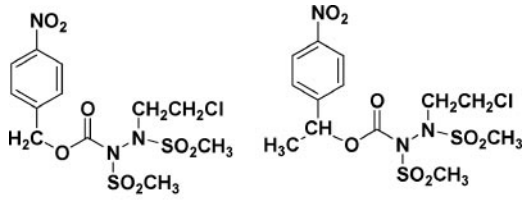

Fig. 1. Structures of PNBC and KS119.

in preclinical systems (20) and has demonstrated significant anticancer activity in patients with relapsed or refractory adult acute myeloid leukemia in recently completed phase I trials (21). This article demonstrates the selectivity of KS119 for hypoxic cells; its activation by xanthine oxidase (XO) and NADPH:cytochrome P450 reductase (NPR); its capacity to crosslink DNA; and its superiority over the related agent, PNBC.

Materials and Methods

Drugs and Chemicals. We synthesized 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119) and PNBC in this laboratory, as described in refs. 13 and 14.

Cell Lines. CHO K1/dhfr$^-$ cells (American Type Culture Collection) were maintained in Iscove's modified Eagle's medium (GIBCO/BRL) supplemented with 10% FBS (Sigma), 2 mM glutamine, 0.1 mM hypoxanthine, and 0.01 M thymidine. Cloned cell lines overexpressing rat NADH:cytochrome b_5 reductase (NBR; 9-fold) (22), human NPR (16-fold) (23, 24), or rat NAD(P)H:quinone oxidoreductase 1 (NQO1; 163-fold) (25) were created by stably transfecting the cDNA for each of these bioreductive enzymes into CHO K1/dhfr$^-$ cells (26). These transfected cells were maintained under the selection pressure of 1 mg/ml G-418 (geneticin; GIBCO/BRL). EMT6 cells were provided by S.R. and grown in Weymouth's medium (GIBCO/BRL) supplemented with 15% of a 1:1 mixture of FBS and fetal clone serum (GIBCO/BRL) and antibiotics. The doubling times for CHO K1/dhfr$^-$ and EMT6 cells were 18 and 14 h, respectively.

Aerobic and Hypoxic Cell Survival Experiments. Cells were seeded in glass milk dilution bottles at 2.5×10^5 cells per bottle and grown for 3 days in a humidified atmosphere of 95% air/5% CO_2. Hypoxia was established by gassing the cultures with a humidified mixture of 95% N_2/5% CO_2 (containing <10 ppm O_2; 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 PNBC and 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% CO_2. Cells were then washed, harvested by trypsinization, and assayed for survival by using a clonogenic assay as described (22, 27).

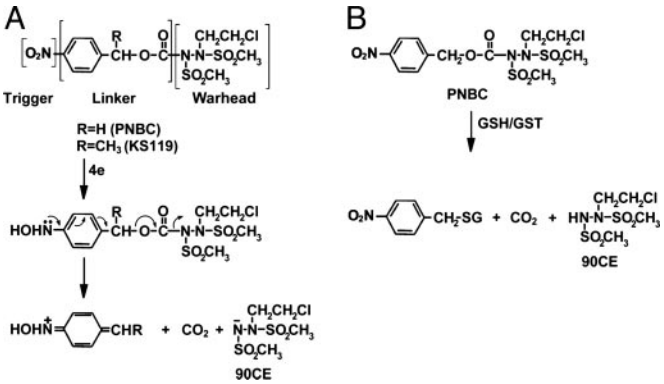

Fig. 2. Schematic representation of the potential activation mechanisms for PNBC and KS119. (*A*) Reductive activation of PNBC and KS119. (*B*) Activation of PNBC by thiolysis.

***In Vitro* DNA Crosslinking.** PNBC or KS119 in 50 mM Tris·HCl/10 mM EDTA (pH 7.0) containing 100 μg/ml T5 DNA (Sigma) was mixed for 1 s with 4 mg/ml Zn dust and allowed to stand at room temperature for 5 min. This mixture results in essentially instantaneous hypoxia because of the reduction of O_2. The Zn was removed by centrifugation, and the supernatant was incubated at 37°C for 15 min to permit the reductively activated drug to form O^6 guanine DNA alkylations. The sample was then incubated for 2 h at 50°C to allow the initial O^6 adducts to proceed to form DNA G–C crosslinks, which require 12–15 h at 37°C to form; the mechanism by which crosslinks are formed from these agents was depicted in ref. 12. The crosslinks were quantified by using an assay based on the snap cooling of DNA in the presence of Hoechst 33258 dye (Molecular Probes) (28).

To determine the half-life of reductively activated drug, Zn dust was incubated with a solution of KS119 for 1 min at room temperature before removing the Zn by centrifugation. The supernatant was then aged at room temperature. Aliquots were removed at various times thereafter and mixed with an equal volume of 200 μg/ml T5 DNA in 20 mM Tris·HCl/1 mM EDTA (pH 7.4). This mixture was then incubated for 15 min at 37°C, followed by 2 h at 50°C. The mixture was then analyzed for crosslinks.

Enzymatic activation of KS119 in the presence or absence of oxygen was determined by using purified human cloned NPR (Sigma). Briefly, a solution containing 200 μM KS119 (added from a 4 mM stock solution in DMSO), 200 μg/ml T5 DNA, 20 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 1 mM NADPH was purged with either N_2 or air for 10 min. NPR (1.1 units/ml) was then added, and samples were incubated for 1 h at 37°C under continuous gas flow. Next, the sample was incubated for 2 h at 50°C, and crosslinks were quantified.

Activation by Glutathione (GSH) and/or GST. The rates of decomposition of various compounds in the presence and absence of GSH and/or GST (Sigma) were determined spectrophotometrically at 560 nm by following the acidification of weakly buffered [1 mM potassium phosphate buffer (pH 7.4) at 37°C] solutions of 20 mg/liter phenol red as described in ref. 14.

HPLC Determination of the Activation of KS119 by Purified NPR or XO Enzymes. Saturated KS119 (18.5 μM, prepared at room temperature) in 20 mM Tris·HCl (pH 7.4) and 1 mM NADPH for studies with NPR or 10 mM acetaldehyde for studies using XO was purged with N_2 or air for 10 min. NPR (1.1 units/ml) or XO (0.1 unit/ml, Sigma) was added and the solution was incubated with purging with either N_2 or air for 1 h at 37°C. Samples in 20 mM Tris·HCl, 1 mM EDTA (pH 7.4) buffer containing 30% acetonitrile were separated on a 5-μm 220 × 4.6-mm C-18 reverse-phase column (RP-18, Applied Biosystems) by elution 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% 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). KS119 eluted as two isomeric conformers in a slightly split single peak at 35 min.

To determine whether both conformers of KS119 were capable of being activated by cellular nitroreductases the above method was modified by eluting the column with a solvent system of constant composition consisting of 39% acetonitrile and

Table 1. Chemical reduction of PNBC and KS119 using Zn/EDTA

Treatment	DNA crosslinks,* %
PNBC	0.37 ± 0.17
PNBC + Zn/EDTA	20.0 ± 0.6
KS119	0.83 ± 0.37
KS119 + Zn/EDTA	21.3 ± 0.9

*Crosslinks were assessed by using a DNA renaturation assay with T5 DNA under conditions of neutral pH as described in *Materials and Methods.* Values represent the means ± SE from three independent experiments.

buffer, at a flow rate of 0.6 ml/min. The conformers eluted as two broad peaks that were readily distinguishable. Also, the concentration of NPR used to activate KS119 was reduced to 0.22 unit/ml, because it was not possible to determine conformer preference if both conformers were reduced completely.

***In Vivo*/*in Vitro* Animal Experiments.** EMT6 tumors were implanted by injecting 2×10^5 cells harvested from exponentially growing cell cultures intradermally in the skin of the rear dorsum of 2.5-month-old BALB/c mice. BALB/c mice bearing established ($\approx$150 mm^3) intradermal EMT6 solid tumors were treated with an i.p. injection of 60–180 mg/kg KS119, followed 100 min later by 15 Gy of total body irradiation using 250-kVp X-rays (15 mA; Al-equivalent filtration, 2 mm; dose rate, 1.2 Gy/min). Next, mice were killed, tumors were excised, single-cell suspensions were prepared, and the survival of the tumor cells was quantified by using a clonogenic assay as described above (22, 27).

Results

Effects of PNBC and KS119 on DNA Crosslinking. PNBC and KS119 were evaluated for differences in crosslinking ability *in vitro* with T5 DNA. In the presence of Zn/EDTA, both PNBC and KS119 formed DNA crosslinks with $\approx$20% of the DNA molecules having one or more crosslinks (Table 1). Since Zn/EDTA readily reduces O_2, generating a hypoxic environment, purging with N_2 was not required. By using purified NPR to activate KS119, the decomposition of this agent depends on prior establishment of a hypoxic environment, a result that is consistent with the finding that KS119 is a hypoxia-selective agent (Table 2). Measurements of the DNA crosslinking activity of KS119 at various times after reductive activation of the compound by Zn/EDTA demonstrate that the decay in crosslinking ability was identical to that of 90CE under the same conditions of room temperature and pH 7.4 (Fig. 3), implying that 90CE is the reactive intermediate generated by the reduction of KS119 as shown in Fig. 2 and that the formation of 90CE from KS119 is relatively rapid and not the rate-determining step in the time course of alkylation.

Effects of PNBC and KS119 on the Survival of EMT6 Cells Under Aerobic and Hypoxic Conditions. The cytotoxic effects of PNBC and KS119 were examined by determining the capacity of EMT6 tumor cells treated under aerobic and hypoxic conditions with either PNBC (Fig. 4*A*) or KS119 (Fig. 4*B*) for 2 h to form colonies. KS119 exhibited considerably greater differential cytotoxicity to EMT6 cells than PNBC, producing 5 logs of kill of cells exposed to 50

Table 2. Enzymatic reduction of KS119 by NPR

Compound	DNA crosslinks,* %
KS119 air	0.1 ± 1.0
KS119 N_2	12.8 ± 1.5

*Enzymatic reduction of KS119 using 1 mM NADPH and 1.1 units/ml purified NPR in air and N_2. Crosslinks in T5 DNA were assessed as described in *Materials and Methods.* Values represent the means ± SE from three independent experiments.

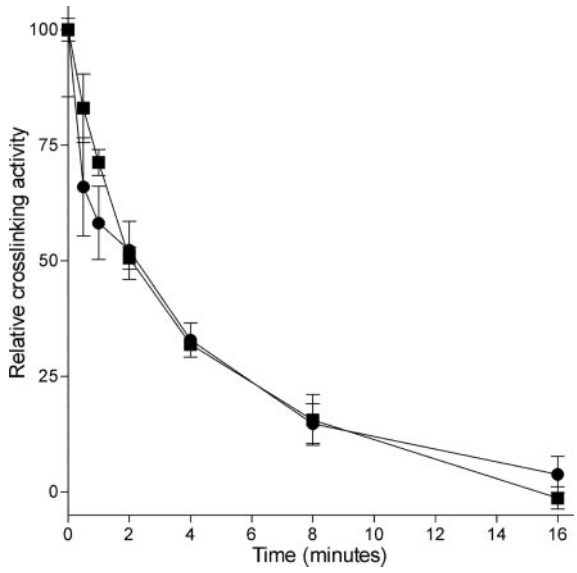

Fig. 3. Comparison of the time course of DNA crosslinking by 90CE (■) and KS119 (●). Solutions containing 90CE and Zn/EDTA activated KS119 were incubated for various periods of time at room temperature and assayed for the ability to crosslink T5 DNA as described in *Materials and Methods*. Values represent the means ± SE from three independent experiments.

μM KS119 in hypoxia for 2 h; whereas, only 2.5 logs of kill were observed in cells exposed to 40 μM PNBC under the same conditions. Under aerobic conditions, the surviving fraction of cells treated for 2 h with 40 μM PNBC was reduced to 0.14 log; whereas no discernable cell kill was observed with cells treated with 50 μM KS119. Thus, little or no aerobic cytotoxicity occurred; however, the finding that some cytotoxicity occurred in PNBC-treated cells under aerobic conditions suggested that PNBC may be activated by secondary mechanisms.

GSH and GST Drug Activation. To determine whether the kill of EMT6 cells by PNBC in air was due to activation by thiolysis, various 2-aryloxycarbonyl-90CE prodrugs were evaluated for activation by a mixture of 5 mM GSH and 17.5 units/ml GST. Activation by hydrolysis and thiolysis depended on the nature of

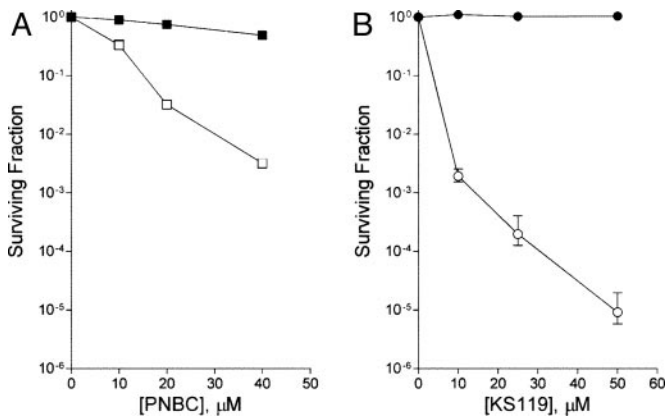

Fig. 4. Comparison of survival curves for EMT6 mouse mammary carcinoma cells treated for 2 h with graded concentrations of PNBC (□, ■) (*A*) and KS119 (○, ●) (*B*) under aerobic (filled symbols) or hypoxic (open symbols) conditions. EMT6 cells were treated as described in *Materials and Methods*. Surviving fractions were calculated by using the plating efficiencies of aerobic and hypoxic vehicle-treated controls within the same experiment. Points represent geometric means ± SE from three independent experiments.

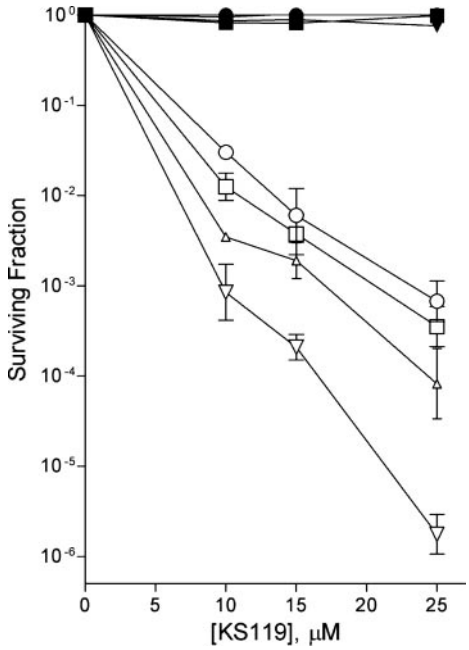

Fig. 5. Survival curves for CHO K1/dhfr$^-$ parental cells (○, ●) and transfectants overexpressing NQO1 [163-fold (□, ■)], NPR [16-fold (▽, ▼)], or NBR [9-fold (△, ▲)] to varying concentrations of KS119 under aerobic (closed symbols) and hypoxic (open symbols) conditions. Cells were treated as described in *Materials and Methods*. Surviving fractions were calculated by using the plating efficiencies of aerobic and hypoxic vehicle-treated controls. Points represent geometric means ± SE from three independent experiments.

the aryloxycarbonyl moiety on the 2-aryloxycarbonyl-90CE backbone, with the addition of an electron-withdrawing group increasing participation in thiolysis reactions, and addition of an electron-donating group greatly diminishing its participation in thiolysis reactions (14). The effects of the addition of a methylene spacer were particularly interesting in that, whereas hydrolysis and thiolysis were reduced as expected because of a weak inductive effect, activation by GST-catalyzed thiolysis of PNBC was markedly increased, with the activation rate being 20.5 nmol·ml·min (14). This phenomenon is likely to be due to a change in the mechanism of activation, whereby a GST catalyzed thiolytic attack occurred at the methylene carbon rather than at the carbonyl carbon, as likely occurred with the other activated compounds of this class lacking the methylene spacer (14). In support of this mechanism is the observation that the addition of a methyl group on the methylene spacer to produce KS119 provided steric hindrance to such an S_N2 attack at the methylene spacer and diminished the GST catalyzed thiolysis to undetectable levels.

Effects of KS119 on the Survival of Parental and Stable CHO Cell Transfectants Overexpressing NBR, NPR, or NQO1. Cells that overexpress NBR, NPR, or NQO1 were examined to gain information on the contributions of these enzymes to the activation and cytotoxicity of KS119; because of difficulties in transfecting EMT6 cells, CHO cells were used for these experiments. The effects of overexpression of NBR (9-fold), NPR (16-fold), and NQO1 (163-fold) on the cytotoxicity of KS119 under aerobic and hypoxic conditions were measured by comparing the survival curves for CHO cell transfectants to those of parental cells (Fig. 5). Overexpression of NBR and NPR, but not of NQO1, increased the kill of CHO cells by KS119 under hypoxic conditions, implying that both NBR and NPR are involved in the activation of KS119, whereas NQO1 is not. Overexpression of NBR, NPR, and NQO1 produced little or no cytotoxicity when

Table 3. Activation of KS119 by NPR and XO in air and N_2

Enzyme	Remaining KS119,* %
NPR + air	97.2 ± 2.7
NPR + N_2	0.002 ± 0.0001
XO + air	100 ± 0.8
XO + N_2	73.5 ± 0.6

The activation of KS119 by 1.1 units/ml of NPR or 0.1 unit/ml XO and 10 mM acetaldehyde was measured by HPLC as described in *Materials and Methods*.
*KS119 was normalized to the amount measured under aerobic conditions before incubation.

cells were exposed to KS119 under aerobic conditions. These findings are consistent with the proposed mechanism in which KS119 generates a cytotoxic species only when it is reductively activated under hypoxic conditions by the initial introduction of single electrons.

Effects of NPR and XO on the Activation of KS119 *in Vitro*. To confirm that the observed increase in cytotoxicity of KS119 in CHO cell transfectants was due to the contribution of one-electron-reducing enzymes, we examined the activation of KS119 by purified NPR and XO in air and hypoxia (Table 3). Activation of KS119 by both NPR and XO occurred only in the absence of oxygen, with 1.1 units/ml purified NPR essentially completely reducing the drug and 0.1 unit/ml purified XO reducing the initial drug concentration by ≈25%, thereby supporting the potential role of these enzymes in the activation of this drug.

Conformational isomers of KS119 were partially separated by HPLC (Fig. 6). To ascertain whether both KS119 conformers were susceptible to reductive activation, we measured the effects of purified NPR and XO on the nitro group trigger. Both conformers were activated almost equally by these one-electron enzyme reductants under hypoxia (Table 4) indicating that both conformational forms of KS119 are approximately equivalent substrates for these enzymes.

Effects of KS119 and X-Rays on EMT6 Tumors in BALB/c Mice. To ascertain whether KS119 has the appropriate physicochemical properties to reach oxygen-deficient cells in solid tumors, an *in vivo/in vitro* assay was used to measure the survival of cells isolated from treated tumors (Table 5). The surviving fractions of cells suspended from established EMT6 tumors in BALB/c mice treated with single i.p. injections of KS119 with and without exposure to x-rays were assessed. Single nontoxic i.p. doses of 60 and 180 mg/kg of KS119 reduced tumor cell survival by 17% and 39%, respectively, demonstrating that activation of KS119 occurred in these tumors. Combined treatment of KS119 (60 or 180 mg/kg) and x-irradiation (15 Gy) had greater than additive

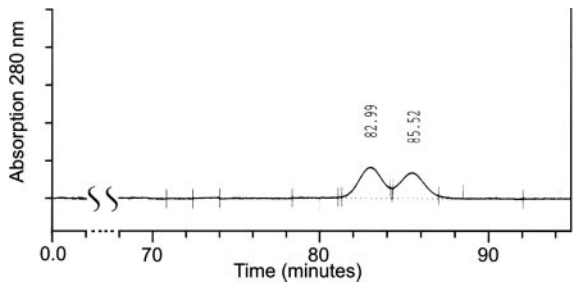

Fig. 6. Separation of KS119 isomeric conformers by HPLC. KS119 conformers were partially separated by using a 5-μm 220 × 4.6 mm C-18 reverse phase column, eluting with a solvent system consisting of 39% acetonitrile in buffer at a flow rate of 0.6 ml/min as described in *Materials and Methods*. The conformers eluted as twin peaks at ≈83 and 85.5 min.

Table 4. Comparison of the enzymatic activation of KS119 isomeric conformers by XO and NPR

Enzyme	% KS119 relative to initial concentration		
	Total*	Conformer 1†	Conformer 2‡
None	100 ± 3	55 ± 1	45 ± 3
XO	78 ± 6	43 ± 3	35 ± 2
NPR	62 ± 9	32 ± 5	30 ± 4

KS119 was activated enzymatically by 0.22 unit/ml human cloned NPR with 1 mM NADPH or 0.1 unit/ml of XO with 10 mM acetaldehyde. Nontransformed KS119 was resolved into individual isomeric conformers using HPLC, quantified and normalized to the total concentration of KS119 present before the addition of enzymes as described in *Materials and Methods.* Values represent means ± SE from three independent determinations.
*Total amount of KS119 was the sum of conformers 1 and 2.
†Conformer 1 was identified by HPLC as a peak that eluted at 83 min.
‡Conformer 2 was identified by HPLC as a peak that eluted at 85.5 min.

effects on tumor cell kill, being ≈5-fold more effective than would be predicted from additive toxicities for individual treatments with KS119 and x-rays. Together, these findings suggest that KS119 is activated by bioreduction in the oxygen deficient regions of tumors, and that the generated species, 90CE, kills large numbers of hypoxic cells and some aerobic cells presumably adjacent to the hypoxic areas.

Discussion

Anticancer prodrugs that require reductive activation to produce cytodestructive species have been useful in the eradication of oxygen-deficient tumor cells; however, a need for more effective hypoxia-selective agents exists. To this end, we synthesized a series of sulfonylhydrazine prodrugs using as the activating trigger the reduction of nitroaromatic compounds (13, 14). Two of these agents, PNBC and KS119 (Fig. 1), were evaluated for their potential as hypoxic-cell cytotoxins. After reductive activation, both of these agents have the capacity to produce the antineoplastic DNA crosslinking species 90CE (19, 20, 29).

Studies evaluating PNBC against EMT6 tumor cells *in vitro* showed approximately a 2.5 log kill of hypoxic cells at a concentration of 40 μM PNBC, with minimal cytotoxicity to aerobic cells (Fig. 4*A*), demonstrating that PNBC has preferential cytotoxicity to hypoxic cells. Although the cytotoxicity of PNBC to EMT6 cells under aerobic conditions was quite low, the kill of some aerobic cells suggested that PNBC was also being activated by a mechanism other than bioreduction of the nitro group (Fig. 2*B*). A previous study of a series of 2-aryloxycarbonyl-90CE with various aryloxycarbonyl substituents demonstrated that agents of this type can be activated by thiolysis under aerobic conditions (14) and the finding that PNBC was readily activated by GST/GSH suggests that GST catalyzed thiolysis may be responsible for the toxicity of PNBC to EMT6 cells under aerobic conditions.

Table 5. Effects of KS119 and x-rays on EMT6 tumors in BALB/c mice

Treatment	Surviving fraction
None	1.00
15 Gy x-ray	0.0380 ± 0.007
60 μg/g KS119	0.83 ± 0.18
180 μg/g KS119	0.61 ± 0.09
60 μg/g KS119 + 15 Gy x-ray	0.0070 ± 0.0001
180 μg/g KS119 + 15 Gy x-ray	0.0045 ± 0.0024

BALB/c mice bearing established tumors (~150 mm^3) were each treated with one i.p. injection of 60 or 180 mg/kg KS119, followed 100 min later by 15 Gy of total body irradiation. Values represent geometric means ± SE of three to five independent experiments.

The large increase in the rate of GST/GSH-dependent activation of PNBC over other synthesized aryloxycarbonyl derivatives implied that the mechanism of activation involved the initiation of an S_N2 attack on the methylene carbon spacer. Because such a mechanism of activation might result in toxicity to normal tissues with relatively high levels of GST, we suppressed activation by such a mechanism to produce a more hypoxia specific drug by the addition of a methyl group onto the methylene carbon spacer to generate KS119 (Fig. 1). Biochemical studies demonstrated that KS119 was not activated by GSH/GST *in vitro*, and in EMT6 tumor cells no aerobic cytotoxicity was produced, whereas ≈5 logs of kill was observed in hypoxic cells, demonstrating that a hypoxia-selective agent was produced (Fig. 4*B*). An analogous level of hypoxic cell kill was also observed in CHO K1/dhfr$^-$ parental cells (Fig. 5) with no discernable toxicity under aerobic conditions, confirming the selectivity of KS119 for hypoxic cells.

Based on the proposed mechanism of activation of KS119 (Fig. 2*A*), reduction of the nitro group is required for decomposition of this agent to produce the cytotoxic species 90CE. The conversion of the nitro substituent of KS119 to a hydroxylamino group probably begins with the input of a single electron, followed by protonation and disproportionation and further reduction as described by Knox *et al.* (16); thus, the initiating enyzmes are one-electron-reducing systems. Under aerobic conditions the input of the initial electron produces the nitro radical anion, which is rapidly scavenged by oxygen (15). This phenomenon results in redox cycling that regenerates the parental prodrug and forms superoxide. In contrast, under hypoxic conditions, the initially formed anion radical accepts other electrons to proceed to the hydroxylamino and/or the amino species, which are capable of spontaneously fragmenting to produce 90CE. To determine which bioreductive enzymes may be involved in the activation of KS119 in intact cells, CHO K1/dhfr$^-$ cells, transfected to overexpress NQO1, NPR, or NBR, were exposed to KS119 under aerobic and hypoxic conditions and cytotoxicity was measured (Fig. 5). Overexpression of NPR and NBR but not NQO1, increased the cytotoxicity of KS119 under hypoxic conditions, whereas these three enzymes did not alter cytotoxicity in air, supporting the concept that NPR and NBR are involved in the activation of KS119. The importance of one-electron-reducing enzymes in the activation of KS119 was directly shown by the finding that purified NPR and XO can serve as catalysts in activating this agent (Table 3) and the fact that activation of KS119 by NPR produces DNA crosslinks (Table 2), arguably the most therapeutically relevant lesion.

The ability of XO to activate KS119 under hypoxic conditions may have importance in the therapeutic activity of KS119 against solid tumors. Whereas most tumor cell types may not contain high levels of XO and xanthine dehydrogenase, endothelial cells express significant levels of these enzymes (30); moreover, hypoxia can increase the activities of these enzymes (31, 32). As a consequence, local antiangiogenic effects may occur, resulting in the killing of vascular tissues residing in the hypoxic regions of the tumor and perhaps also in aerobic tumor cells adjacent to this vasculature through bystander effects.

By using HPLC, we have been able to resolve two isomeric conformational forms of KS119 to demonstrate that both conformers of KS119 are reduced essentially equally by the one-electron-reducing enyzmes, NPR and XO. This action initiates the activation process; therefore, it is reasonable to assume that both conformers are therapeutically active.

The $T_{1/2}$ of the reductively activated KS119 and PNBC is important for targeting hypoxic cells. Reduced species with relatively long half lives might compromise the targeting ability

of the drug, because the activated agent might distribute throughout the body. Although long-lived species would show potent hypoxic cell selectivity in cell-culture tests, they might be ineffective at targeting hypoxic regions *in vivo*. To ascertain the stability of the reductively activated species, we used an *in vitro* assay in which Zn/EDTA was used to (*i*) create rapid hypoxia and (*ii*) reductively activate PNBC and KS119. Because the reduction of PNBC and KS119 by Zn/EDTA at room temperature was essentially complete within 1 min, it allowed the performance of experiments to quantify the decay of crosslinking activity of KS119 after activation; a $T_{1/2}$ value for KS119 of ≈2.5 min was obtained at room temperature. The loss of crosslinking activity as a function of time, exactly matched that found when the experiment was conducted using 90CE in place of KS119. This finding implied that the reductively activated KS119 decomposed quickly to form 90CE, such that the decomposition of 90CE was rate determining with respect to the loss of alkylating activity. 90CE, which has a $T_{1/2}$ of ≈30 s at 37°C and pH 7.4, would likely show a good degree of precise targeting because diffusional loss of the drug from the site of activation would be minimal within this window of time. Because the 90CE moiety appears to be the rate-determining step in the loss of alkylating activity after reductive activation and the $T_{1/2}$ of agents of this type are easily manipulated by changing the electron withdrawing and donating power of the N-1 and N-2 substituents (33), it would be relatively easy to design prodrugs generating 90CE-like agents with greater or lesser diffusional radii from the site of activation.

An important issue for a hypoxic cell selective agent is that the agent possesses physicochemical properties that permit it to reach hypoxic areas in solid tumors. The data presented in Table 5 on the effects of KS119 in mice bearing solid EMT6 tumors, with and without radiation, demonstrate that KS119 has the appropriate physicochemical properties to reach hypoxic tumor regions and to kill oxygen-deficient cells located in these areas. The findings collectively demonstrate that KS119 is a hypoxia-selective cytotoxic agent; furthermore, its selective cytotoxicity to hypoxic tumor cells coupled with its lack of toxicity to well oxygenated cells and, therefore, presumably normal tissue, make KS119 a worthy candidate for a clinical trial.

This work was supported in part by National Cancer Institute U.S. Public Health Service Grant CA-90671.

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