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KS900: a hypoxia-directed, reductively activated methylating antitumor prodrug that selectively ablates O⁶-alkylguanine-DNA alkyltransferase in neoplastic cells

Raymond P. Baumann, Kimiko Ishiguro, Philip G. Penketh, Krishnamurthy Shyam, Rui Zhu, and Alan C. Sartorelli*

Department of Pharmacology and Cancer Center, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06520

Abstract

To most effectively treat cancer it may be necessary to preferentially destroy tumor tissue while sparing normal tissues. One strategy to accomplish this is to selectively cripple the involved tumor resistance mechanisms, thereby allowing the affected anticancer drugs to gain therapeutic efficacy. Such an approach is exemplified by our design and synthesis of the intracellular hypoxic cell activated methylating agent, 1,2-bis(methylsulfonyl)-1-methyl-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS900) that targets the O-6 position of guanine in DNA. KS900 is markedly more cytotoxic in clonogenic experiments under conditions of oxygen deficiency than the non-intracellularly activated agents KS90, and 90M, when tested in O⁶-alkylguanine-DNA alkyltransferase (AGT) non-expressing cells (EMT6 mouse mammary carcinoma, CHO/AA8 hamster ovary, and U251 human glioma), and than temozolomide when tested in AGT expressing cells (DU145 human prostate carcinoma). Furthermore, KS900 more efficiently ablates AGT in HL-60 human leukemia and DU145 cells than the spontaneous globally activated methylating agent KS90, with an IC₅₀ value over 9-fold lower than KS90. Finally, KS900 under oxygen-deficient conditions selectively sensitizes DU145 cells to the chloroethylating agent, onirgin, through the ablation of the resistance protein AGT. Thus, under hypoxia, KS900 is more cytotoxic at substantially lower concentrations than methylating agents such as temozolomide that are not preferentially activated in neoplastic cells by intracellular reductase catalysts. The necessity for intracellular activation of KS900 permits substantially greater cytotoxic activity against cells containing the resistance protein O⁶-alkylguanine-DNA alkyltransferase (AGT) than agents such as temozolomide. Furthermore, the hypoxia-directed intracellular activation of KS900 allows it to preferentially ablate AGT pools under the oxygen-deficient conditions that are present in malignant tissue.

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*Corresponding author: Raymond P. Baumann, raymond.baumann@yale.edu, Tel: 203-785-4524, FAX: 203-785-2494.

Disclosure

Conflict of Interest Statement

The potential anticancer agents OnirginTM and KS119, designed and synthesized in Dr. Sartorelli's laboratory, were licensed to now bankrupt Vion Pharmaceuticals, Inc. by Yale University which now has consequently regained them. Dr. Sartorelli was a Director and Chairman of the Scientific Advisory Board of this company, had common stock in Vion and, in the past, his laboratory has.. received gift monies in support of research. Presently there is no conflict of interest by Dr. Sartorelli or members of his laboratory.

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Keywords

Oxygen-deficient cells; O⁶-Alkylguanine-DNA alkyltransferase; 1, 2-Bis(sulfonyl)hydrazines; KS900; OnriginTM

1. Introduction

The blood supply of solid tumors can be haphazard and poorly developed resulting in chronic areas of limited or absent oxygen availability within these tumors [1–4]. While hypoxic/anoxic regions may alter tumor development, potentially leading to a more aggressive neoplastic phenotype [5], the reductive environments present within oxygen-deficient tumor areas are also prospective sites for chemotherapeutic attack through the preferential or selective activation of hypoxia targeted antitumor agents.

The tissue response to fluctuations in oxygen levels is complex and is carefully orchestrated through an interplay between Hif-1, angiogenesis inducers, and other molecular factors involved in sensing oxygen levels [6–10], but these processes may be hijacked and manipulated by migrating neoplastic cells to facilitate metastatic spread of tumors [10–12], suggesting that oxygen deficiency may be an essential component in seeding new neoplastic sites. Thus, another potential benefit of agents that target hypoxic tumor tissue might be the capacity to prevent or diminish the tumor seeding of metastatic sites.

We have previously described the design, synthesis and preclinical evaluation of a DNA cross-linking agent, KS119, which is activated preferentially within the reductive enzymatic environment present in oxygen-deficient tumor regions [13–16]. This agent, upon activation by one-electron reductase enzymes in oxygen-deficient tumor cells, produces the same cytotoxic chloroethylating product as the clinically active prodrug onrigin (laromustine, cloretazine, VNP40101M, 101M) [17–23]. This agent, which chloroethylates the O-6 position of guanine in DNA that ultimately results in the formation of a G-C interstrand DNA cross-link. However, the effectiveness of such agents is mitigated by the expression of the resistance protein O⁶-alkylguanine-DNA alkyltransferase (AGT) in many cancers [24–27]. AGT is a protein which removes alkyl groups from the O-6 position of guanine in DNA by transferring them to cysteine-145 within its active site, thereby restoring the DNA to its native state. This process is stoichiometric and irreversible, resulting in the inactivation and ultimately the degradation of the AGT protein; the only known mechanisms of regeneration for AGT is through the direct re-synthesis of the protein [24, 25, 27].

Efforts to inactivate AGT in cancer tissue and enhance the efficacy of O-6 guanine alkylating agents in patients have not been particularly successful because systemic AGT depleting agents also sensitize normal tissue that contain protective levels of AGT, thereby eliminating any AGT expression differential between the tumor and normal tissue [27–29]; such action requires marked dose reduction of the alkylating agent to prevent severe treatment toxicity, resulting in a non-therapeutic dose level of drug. Hence, a complementary approach to developing a highly cytotoxic targeted cross-linking agent such as KS119 or a less selective agent such as onrigin, would be to develop an intracellular hypoxia activated inhibitor of AGT that preferentially ablates AGT in neoplastic tissue. Such a strategy has the potential to sensitize tumors to a spectrum of chemotherapeutic agents that alkylate the O-6 position of guanine in DNA and are subject to repair by AGT, thereby broadening the range not only of tumors that could be treated but also of drugs that may be employed. To this end we have utilized the KS119 nitrobenzyloxycarbonyl trigger-linker platform to synthesize a methylating agent, 1,2-bis(methylsulfonyl)-1-(2-methyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS900), which is designed to be

preferentially activated in tumor tissue under oxygen-deficient conditions. KS900 is envisioned to preferentially deplete AGT under oxygen-deficient conditions, and thereby selectively sensitize tumors to O-6 guanine alkylating agents such as ongrin, carmustine, lomustine, temozolomide, procarbazine, and dacarbazine.

KS900 is a prodrug designed to be intracellularly activated by one electron reductive enzymes under oxygen-deficient conditions found in tumor tissue. The requirement for intracellular prodrug activation concentrates the active methylating agent within or near the cell likely resulting in a substantially greater number of methyl lesions to cellular macromolecules including DNA than produced by non-intracellularly activated prodrugs which spontaneously activate both inside and outside targeted cells. This increased delivery is facilitated by short half lives of the active intermediates which limits diffusion and results in greater ablation of AGT and greater cytotoxicity when this resistance protein is not present or has been completely ablated.

2. Materials and methods

2.1. Cell lines

CHO/AA8 Chinese hamster ovary, EMT6 murine mammary carcinoma, and U251 human glioblastoma cells which lack AGT (expression is below the level of detection of less than 600 AGT molecules/cell) and DU145 prostate carcinoma cells (containing 42,000 AGT molecules/cell) were maintained under 5% CO₂ in α -MEM (CHO/A88, U251 and DU145 cells) or DMEM (EMT6 cells) supplemented with 10% FBS. HL60 human leukemia cells were propagated in suspension in RPMI with 10% FBS. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Cell line AGT amounts were determined using a previously described [³H]O⁶-benzylguanine binding assay [30].

2.2. Chemical syntheses

The 1,2-bis(sulfonyl)hydrazine prodrugs were synthesized by previously published procedures [14, 31]. 1,2-Bis(methylsulfonyl)-1-methyl-2-[[1-(4-nitrophenyl)ethoxycarbonyl]hydrazine (KS900) was synthesized using the following strategy: To an ice-cooled solution of 1-(4-nitrophenyl)-1-ethoxycarbonyl chloride (1.27 g, 5.5 mmol) in pyridine (2 mL) was added 1,2-bis(methylsulfonyl)-1-methylhydrazine (KS90; 1.10 g, 5 mmol) at 0 °C. The mixture was stirred at the same temperature for two additional hours and then left in a freezer for 24 h. The resulting mixture was diluted with water (50 mL), acidified with concentrated hydrochloric acid to pH 4–5, and extracted with CH₂Cl₂ (3 × 50 mL). The organic layer was washed with water and brine, and dried over anhydrous sodium sulfate. Removal of the solvent yielded the crude product, which was purified by column chromatography (DCM: EtOAc = 8:1) to give 870 mg (44%) of KS900 as a white solid; m.p. 132–134 °C (DCM-EtOH); ¹H NMR: δ 8.29–8.22 (m, 2H), 7.62–7.57 (m, 2H), 6.04–5.96 (m, 1H), 3.41 (d, *J* = 5.2 Hz, 3H), 3.34 (d, *J* = 8.5 Hz, 3H), 3.11 (d, *J* = 3.6 Hz, 3H), 1.69 (d, *J* = 6.6 Hz, 3H); ¹³C NMR: δ 151.4, 151.3, 148.0, 147.9, 146.9, 146.7, 127.0, 126.8, 124.2, 124.0, 42.0, 41.9, 40.3, 40.2, 39.0, 38.9, 22.5, 22.1. Anal (C₁₂H₁₇N₃O₈S₂) C, H, N.

Preparation of 1,2-bis(methylsulfonyl)-1-methyl-2-(methylamino)carbonylhydrazine (90M): To a stirred mixture of 1,2-bis(methylsulfonyl)-1-methylhydrazine (KS90; 1.01 g, 5 mmol) and acetone (2 mL) was added methyl isocyanate (0.4 g, 7 mmol), followed by 5 drops of triethylamine. The reaction mixture was stirred at room temperature for 2 h. It was then evaporated to dryness in vacuo, the residue taken up in chloroform (100 mL) and washed with 10% hydrochloric acid (15 mL). The chloroform layer was dried over anhydrous sodium sulfate, filtered and evaporated to dryness to give a solid, which was recrystallized

from anhydrous ethanol to give 0.63 g (48.6 %) of the title compound (m.p. 119–120 °C). ¹H NMR: δ 6.42 (br, 1H), 3.34 (s, 3H), 3.37 (s, 3H), 3.21 (s, 3H), 2.89–2.90 (d, 3H). Anal. (C₅H₁₃N₃O₅S₂) C, H, N. Elemental analysis of KS900 and 90M indicated that both compounds were within 0.4% of theoretical values. All other drugs and chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO).

2.3. Toxicity studies

Cell survival (clonogenic) assays were performed using a previously described method [19]. Twenty-five cm² plastic tissue culture flasks were seeded with 5 to 8 × 10⁵ cells each and when confluent, cells were treated with the desired agents dissolved in 10 ml of medium for 24 h at 37°C. All agents were dissolved first in DMSO with the exception of temozolomide which was dissolved in medium at a concentration of 10 mM and diluted to the needed concentration. For oxygen-deficient conditions, cells were incubated with 1,2-bis(sulfonyl)hydrazines in the presence of 2 Units(U)/ml of glucose oxidase (Sigma G6641), 120 U/ml of catalase (Sigma, C1345) in high glucose DMEM (Invitrogen) [16, 32]. Flasks were flushed with nitrogen for 10 s and the caps screwed on tightly. This facilitates oxygen depletion of the medium by glucose oxidase through removal of residual oxygen containing air and denial of the entry of additional air. After treatment, monolayers were rinsed, and cells were detached by trypsinization, suspended in culture medium, counted and sequential cell dilutions were plated in duplicate into 6-well plates at a density of 1 × 10², 1 × 10³, or 1 × 10⁴ cells per well. Seven to ten days later, colonies were fixed, stained with crystal violet (0.25%) in 80% methanol and quantified. For studies involving sensitization to onirigin, cells were pretreated for 4 h in the presence of the desired methylating agent prior to the addition of onirigin for a total incubation time of 24 h. All analyses were corrected for plating efficiency in the presence of vehicle (DMSO) at concentrations equivalent to those used for exposure to the test 1,2-bis(sulfonyl)hydrazine. DMSO concentrations were ≤0.05%, and non-toxic. Cells under aerobic conditions were treated under similar conditions and cytotoxic agent concentrations, but in unsealed flasks without glucose oxidase and catalase. Cells were then washed, harvested by trypsinization, and assayed for survival using a clonogenic assay described previously [19, 33–35]. In the absence of cells, no measurable direct metabolism of KS900 was detected in the presence of the glucose oxidase and catalase enzyme components of our oxygen deficiency generating system [32].

2.4. HPLC determination of the disappearance of KS900 in cell cultures under conditions of oxygenation and oxygen deficiency

For these experiments, 10⁷ cells/ml were incubated for various times with KS900 under oxygenation or enzyme generated oxygen deficiency described above. For studies under oxygenation, cells were incubated in 25 cm² flasks in shallow 5 ml layers of growth medium containing 10% FBS (α-MEM for DU145 cells or DMEM for EMT6 cells) with shaking at 37 °C. Experiments under oxygen-deficient conditions were performed in sealed 1.5 ml tubes in the presence of 2 U/ml of glucose oxidase (Sigma G6641), 120 U/ml of catalase (Sigma C1345) and 10 mM added glucose in 1 ml of growth medium. Cell supernatant samples containing KS900 were mixed at various incubation times 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 micron 220 mm × 4.6 mm C-18 reverse-phase column (RP-18, Applied Biosystems); elution was accomplished with 34.5% acetonitrile in buffer (0.03 M KH₂PO₄/1.0 mM NaN₃, 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). KS900 eluted at approximately 28.8 min.

2.5. AGT assays

AGT assays were performed essentially as described by Ishiguro et al. [30]. Determination of AGT activity relied upon stoichiometric covalent transfer of radioactive benzyl residues from [benzene-³H]O⁶-benzylguanine to AGT, and the numbers of AGT molecules/cell were calculated based upon radioactivity in a 70% methanol precipitable fraction, the specific activity of [benzene-³H]O⁶-benzylguanine and Avogadro's number as described [30].

2.6. AGT ablation studies

For AGT ablation studies in HL60 cells, packed cells at a concentration of 2×10^7 cells/ml were treated at 37°C at incremental times with KS90 or KS900 then AGT levels were quantified by the [benzene-³H]O⁶-benzylguanine AGT binding method [30]. AGT ablation with KS900 in DU145 cells was performed as follows. Confluent monolayers of cells in 75 cm² flasks were treated for 4 h or 24 h in 30 ml of high glucose DMEM under conditions of oxygenation or enzymatic generated oxygen deficiency [32] with 200 μM of KS900 at 37°C. Cells were detached by trypsinization and the AGT levels determined [30].

3. Results

3.1. Comparative methylator toxicity in cells lacking AGT

KS900 is an intracellular targeted methylating agent designed to be activated by one electron cellular reductase enzymes (NADPH: cytochrome p450 reductase, NADH: cytochrome b5 reductase and/or xanthine reductase) under conditions of oxygen deficiency where back oxidation of the initially generated nitro radical ion to the parent prodrug does not occur. We compared the cytotoxicity of KS900 to two other methylating agents, 1,2-bis(methylsulfonyl)-1-methylhydrazine, KS90, and 1,2-bis(methylsulfonyl)-1-methyl-2-(methylamino)carbonylhydrazine, 90M, both of which are not activated specifically within tumors and therefore not designed to be preferentially activated under oxygen-deficient conditions (Fig. 1A, B). All three of the synthesized agents were designed and produced in our laboratory and each generated the same ultimate active methylating agent upon decomposition. For toxicity tests, three AGT deficient cell lines were used, CHO/AA8 hamster ovary cells, U251 human glioma cells, and EMT6 murine mammary carcinoma cells. As shown in Fig. 2B, 2D, and 2F under conditions of oxygen deficiency, KS900 was substantially more cytotoxic than KS90 or 90M at equimolar concentrations in all three cell types. As expected for this oxygen deficiency selective agent, little toxicity was seen with KS900 under oxygenated conditions (Fig. 2A, 2C, and 2E). These findings emphasize the advantage of the intracellular targeting of methylating agents by requiring the active alkylation product to be formed through cellular enzymatic reduction under conditions of oxygen deficiency. Thus, under oxygen-deficient conditions in cells lacking AGT expression, KS900 can exert cellular toxicity by methylating the O6-position of guanine in DNA.

In general, methylating agents are weakly cytotoxic compared to the more potent chloroethylating cross-linking alkylating agents exemplified by ongrin and carmustine [36]. While KS90 and 90M generate a similar active metabolite to KS900, their lack of intracellular activation results in little or no cytotoxicity at the concentration range used in Figure 2, but as demonstrated in Figure 3C when used at much higher concentrations KS90 does exhibit cytotoxicity. The half lives of KS90 and 90M under aqueous conditions are 2.8 min and ≈1 h, respectively. KS900 decomposes under similar conditions much more slowly, at about 10–20% every 24 h. In the cell toxicity assay conditions employed in the present studies, the rate of active cellular metabolism of KS900 cannot be distinguished from its spontaneous decomposition. These additional observations reinforce the utility of required intracellular activation to produce the cytotoxic lesions directly in the neoplastic cell.

3.2. Comparative methylator toxicity in cells expressing AGT

Surprisingly, studies measuring the solubility of KS900 under aqueous conditions, indicated that it is markedly more water soluble (752 μM at 37°C) than the chloroethylating version, KS119 (solubility less than 50 μM at 37°C) [16]. This difference allowed us to evaluate KS900 at effective concentrations against the AGT expressing human prostate carcinoma cell line DU145, which contains an average of 42,000 AGT molecules per cell [30]. As shown in Fig. 3A, KS900 exhibited a marked oxic/hypoxic differential in DU145 cells, being active under oxygen-deficient conditions while displaying little or no cytotoxic activity under oxygenation. This is in contrast to the non-selective agents temozolomide (Fig. 3B) and KS90 (Fig. 3C) which show little or no differential as a function of oxygen concentration. Furthermore, as shown in Fig. 3D, KS900 demonstrates little or no cytotoxicity under oxygenation, but is markedly more cytotoxic at lower concentrations under conditions of oxygen deficiency than KS90 or temozolomide (Fig. 3E).

3.3. Metabolism and decomposition of KS900

The metabolism of KS900 was studied in DU145 (Fig. 4A) and EMT6 (Fig. 4B) cells. These experiments were performed using high cell densities which may better represent the cellular densities exhibited by tumor masses than single cell monolayers [16, 30]. KS900 was incubated with cells at a density of $10^7/\text{ml}$, and samples were analyzed using HPLC at 1 h intervals for a total of three hours. As shown in Fig. 4A and 4B, KS900 was metabolized to a significantly greater extent under oxygen-deficient conditions in both cell lines at all time points. These experiments measured the disappearance of KS900, the assumption being that the disappearance of KS900 represented activation of the prodrug to produce the methylating agent KS90. This was explored using the AGT ablation studies described subsequently. The half life of KS90 is only 2–3 minutes and its production by the prodrug KS900, which is predicted to occur at a low steady-state level, cannot be measured by HPLC; however, we have demonstrated the production of KS90 indirectly by the ablation of AGT (Figures 5 and 6) and also by the production of methanol after chemical reaction of KS90 with water (unpublished observation).

3.4. Ablation of AGT by KS900

One of the most promising design features of KS900 is the potential for targeted intracellular inactivation of AGT through the methylation of cellular DNA by its active metabolite, KS90, and the subsequent ablation of AGT through the stoichiometric repair of O-6 methylguanine by the suicide repair protein AGT. Since methylated DNA is the preferred natural substrate for AGT, its generation is an exceedingly effective way to deplete AGT. Furthermore, since AGT is not an enzyme and, therefore not catalytic, it does not get regenerated through any known cellular mechanism. Thus depleted AGT must be resynthesized in order to reconstitute its repair potential, which is a time consuming process with a substantial delay [27].

We have compared the ability of KS900 and KS90 to deplete AGT in HL60 human leukemia cells at high cell densities. Oxygraph measurements have indicated that under high cell densities and small sample volumes oxygen is rapidly depleted by cells in under 15 min, essentially rendering the sample oxygen-deficient and eliminating the need to employ glucose oxidase to directly deplete oxygen. As shown in Fig. 5, under these conditions, the slowly activated intracellular KS900 depletes AGT as effectively as the relatively rapid non-selectively activated KS90 at 1 h, but is much more efficacious (almost ten-fold) at the longer incubation time point of 4 h. This enhanced ability to deplete AGT, despite the more prolonged and incomplete activation of KS900, is likely the result of its intracellular activation paralleling its greater cytotoxicity than the non-selectively activated agents KS90 and 90M whose activation occurs both outside and inside tumor cells. Similar results, for

high cell density AGT depletion were seen with DU145 prostate carcinoma cells (data not shown).

Additional AGT ablation studies were performed using attached monolayers of DU145 cells under conditions similar to those employed for clonogenic assays. Cells were treated for 4h (Fig. 6A) or 24 h (Fig. 6A) using 200 μ M KS900 under either oxygen-deficient or oxygenated conditions. As observed in Fig. 6, using cell monolayers, substantial AGT depletion occurred under conditions of oxygen deficiency after both 4 and 24 h incubation times, at levels approaching those found in the presence of a large excess (250 μ M) of the potent AGT inhibitor O⁶-benzylguanine (Fig. 6A). Although substantial AGT depletion by KS900 occurred at 24 h under oxygenated conditions (Fig. 6B), this prolonged time dependent depletion did not sensitize cells to onrigin (Fig. 7). The O⁶-BG sample represented essentially complete ablation of AGT; however, residual radioactivity was present even after treatment with a vast excess of non-radiocative O⁶-BG because of non-specific binding by this labeled compound.

3.5 KS900 enhancement of onrigin lethality

One of the greatest predicted benefits of intracellular activated methylating agents that selectively deplete AGT in oxygen-deficient regions of neoplastic tissue, is the potential to subsequently confer therapeutic efficacy to non-targeted agents or drugs, particularly chloroethylators which alkylate the O-6 position of guanine in DNA for which AGT is a major resistance factor controlling therapeutic response. We evaluated the ability of KS900 to preferentially augment the cytotoxicity of onrigin under conditions of oxygen deficiency compared to the non-selectively activated agents KS90 and temozolomide, which have the potential to be activated both extra- and intracellularly. As shown in Figure 7A and 7B, KS900 shows a clear cytotoxic differential when combined with onrigin in oxygen depleted DU145 cells which express AGT (Fig. 7A), but not under conditions of oxygenation (Fig. 7B). This finding is in contrast to KS90 (Fig. 7C and 7D) and temozolomide (Fig. 7E and 7F) which show no selectivity for activation under conditions of oxygen deficiency.

As indicated in Figure 3D and E and Figure 7, the cytotoxicity of the prodrugs KS900, KS90, temozolomide and onrigin when used alone on DU145 cells was markedly less than when used in combination with each other. DU145 cells treated with onrigin, at a concentration of 100 μ M, the maximum concentration employed in these studies, resulted in cell survival values of 71% and 61 % under oxygenated and oxygen-deficient conditions, respectively. However when DU145 cells were exposed to onrigin (100 μ M) in combination with KS90 (1.5 mM) the maximum employed concentration exhibited a 67-fold (53.5 to 0.8%, oxygenated) and a 420-fold (38% to 0.09%, oxygen-deficient) decrease in survival; onrigin in admixture with temozolomide (1.5 mM) demonstrated a 15-fold (22% to 1.5%, oxygenated) and 740-fold (22.3% to 0.03%, oxygen-deficient) decrease in survival, and onrigin in combination with KS900 (at 200 μ M) a 1400-fold (28.4% to 0.02%, oxygen-deficient) decrease in survival, than in the absence of onrigin. Under oxygenated conditions DU145 cells treated with KS900 alone exhibited a 100% survival, whereas in combination with onrigin 62% survival, which was not significantly different from that of onrigin alone (71%), was observed. Thus, KS900 was substantially more effective under oxygen-deficient conditions, requiring much less drug to maximally enhance the cytotoxicity of onrigin than methylators not requiring intracellular enzymatic activation, while showing no enhancement under oxygenated conditions.

4. Discussion

KS900 is an methylating agent prodrug designed to be similar to the chloroethylating agent KS119 by its utilization of the nitrobenzyloxycarbonyl trigger/linker platform employed

with KS119 (14–16, 37). This leads to the generation of the cytotoxic warhead directly inside neoplastic cells following reductive activation by one electron cellular reductase enzymes under conditions of oxygen deficiency, an action that results in subsequent alkylation of the O-6 position of guanine in DNA. These design features confer upon KS900 the potential to selectively deplete AGT in oxygen-deficient regions of tumors, thereby rendering them susceptible to other more cytotoxic prodrugs to which AGT provides resistance. Findings presented in this report demonstrate that KS900 is preferentially activated under conditions of oxygen deficiency in several cell lines, in a manner analogous to that of the chloroethylating hypoxia/anoxia targeted prodrug KS119 (16). The findings also suggest that the enzymes responsible for reductive activation of KS900 are not restricted to certain tumor cell lines and thus appear to be ubiquitous.

In cells which lack AGT, KS900 is more cytotoxic than non-intracellular activated methylating agents such as temozolomide and KS90 under oxygen-deficient conditions at equivalent concentrations. We speculate that the structurally directed intracellular activation of KS900 in tumors combined with the stability of this agent under aqueous conditions results in more efficient, total and prolonged delivery to both DNA and other cellular macromolecules of an activated methylating agent which yields an increase in cellular alkylation compared to non-directed agents. This advantage also extends to AGT expressing cells such as the human prostate carcinoma cell line DU145, which contains an average of 42,000 molecules of AGT/cell, where KS900 exhibits greater cytotoxicity under oxygen-deficient conditions than non-intracellular only activated prodrug methylators. Perhaps, the most important finding concerning KS900 is its capacity to selectively deplete AGT in cells expressing this repair protein under conditions of oxygen deficiency.

Clinical studies in humans with global AGT depleting agents such as O⁶-BG in combination with a cross-linking agent such as carmustine have failed to demonstrate any clinical benefit to cancer patients because O⁶-BG is a non-targeted agent that also ablates AGT in normal tissues; this necessitates a major reduction in the dose of carmustine to prevent unacceptable toxic side effects that renders this agent non-active [27, 28]. Thus, KS900, may permit a targeted depletion of the DNA repair protein AGT in tumor tissue, thereby not only providing cytotoxic kill of neoplastic cells but also sensitization of the tumor to non-targeted agents which alkylate the O-6 position of guanine in DNA. Further studies are necessary to determine if clinically relevant concentrations of KS900 can be achieved.

There is increasing evidence that oxygen-deficient niches are important in the metastatic seeding of cancers [10–12]. Thus, another potential benefit of agents that undergo activation selectively in oxygen-deficient areas of tumors is the possibility that they will interfere with the metastatic process. In this regard, KS900 would seem to be particularly appealing because of its dual ability to both exert cytotoxicity, as well as sensitize cells to more potent cytotoxic alkylating agents that cause O⁶-chloroethylation. It is important to note that the efficacy of any agent designed to be selectively activated within hypoxic/anoxic tumor regions may be influenced by the tumor vascular architecture and cellular composition. The prevalence and temporal duration of areas of tumor oxygen deficiency may vary substantially among different cancers thereby strongly influencing any therapeutic effect exerted by agents targeting the oxygen deficiency when used as a single treatment modality. It would seem unreasonable to assume that these agents can sensitize an entire tumor when only a subset of the neoplasm contains oxygen-deficient cells. Hence, it is important to consider strategies to maximize bystander effects/kill with these agents and to find drugs/treatments which enhance their action, which may include combining them with radiation to deplete oxygen in tumors (15, 34), as well as with other drugs that can kill oxygenated tumor cells or that can alter tumor vascularization such as angiogenesis inhibitors, in order to fully reveal the therapeutic potential of drugs targeting oxygen deficiency.

Other methods of potentially targeting tumor cells with AGT inhibitors have been described. These include conjugating AGT depleting agents such as O⁶-BG to metabolites that are highly sought by tumor cells, such as folate or glucose (38, 39), as well as direct localized treatment of tumors with AGT inhibitors (for a review see ref [27]). The delivery platform described in this report may prove sufficiently versatile for utilization with other molecules that potentially inactivate AGT, in addition to the oxygen deficiency directed methylating agent, KS900.

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Abbreviations

AGT	O ⁶ -alkylguanine-DNA alkyltransferase
90CE	1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine
90M	1,2-bis(methylsulfonyl)-1-methyl-2-(methylamino)carbonylhydrazine
KS900	1,2-bis(methylsulfonyl)-1-methyl-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine
KS119	1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine
OnriginTM	1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-(methylamino)carbonylhydrazine
O⁶-BG	O ⁶ -benzylguanine

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Fig. 2.

Clonogenic experiments using CHO/AA8, U251 and EMT6 cell lines treated with various agents under conditions of oxygenation or oxygen deficiency. CHO/AA8 cells (oxygenated, panel A; oxygen-deficient, panel B), U251 cells (oxygenated, panel C; oxygen-deficient, panel D) and EMT6 cells (oxygenated, panel E; oxygen-deficient, panel F) were exposed to incremental concentrations of KS900 (▲), KS90 (■), or 90M (▼). The Y axis indicates the percent survival; the X axis indicates the concentration of the agents employed. For oxygen-deficient samples, oxygen was depleted enzymatically in the presence of 2 U/ml of glucose oxidase, 120 U/ml of catalase and 25 mM glucose, for 24 h, prior to measurement of cloning efficiency. All experimental points represent at least 3 independent determinations $\pm$ S.E.M.

Fig. 3.

DU145 AGT expressing cell survival following treatment with KS900, KS90, or temozolomide under oxygenation or oxygen deficiency. DU145 cells were exposed to increasing amounts of KS900 (panel A), temozolomide (panel B), or KS90 (panel C) under conditions of oxygenation (■) or oxygen deficiency (▲). Combined graphs for, KS900 (▲), KS90 (■), or temozolomide (▼) under oxygenated (Panel D) or oxygen-deficient conditions (Panel E). The Y axis indicates the percent survival; the X axis indicates the concentration of the agents employed. Oxygen was removed from test samples enzymatically in the presence of 2 U/ml of glucose oxidase, 120 U/ml of catalase and 25 mM glucose, before harvesting and plating of cells to measure cloning efficiency. All points are the result of at least three independent experimental determinations $\pm$ S.E.M.

Fig. 4.

Activation of KS900 at high cell densities using DU145 and EMT6 cells. Cells (DU145, panel A; EMT6, panel B) at 10^7 per ml were exposed to 20 μM KS900 for various periods of time either under oxygenation (■) or oxygen deficiency (▲). Cells were lysed by mixing with an equal volume of acetonitrile which also served to quench all reactions, extract drug, and precipitate proteins. Lysed cell/acetonitrile mixtures were incubated for 15 min at room temperature, centrifuged at $10,000 \times g$ for 5 min, and the supernatants were analyzed by HPLC. The Y axis indicates KS900 concentrations as measured by absorbance at 280 nm. Incubation time is given on the X axis. All points are the result of at least three independent experimental determinations $\pm$ S.E.M.

Fig. 5.

AGT depletion by KS90 and KS900 under oxygen deficiency. HL-60 cells (2×10^6 cells/100 μl) were exposed to inhibitors for 1 h or 4 h, then AGT was quantified in the presence of 1 μCi of [benzene- ^3H]O 6 -benzylguanine [30]. IC_{50} values represent the mean $\pm$ S.E.M. from 3 independent experiments.

Fig. 6. AGT depletion by KS900 under oxygen deficiency and oxygenation in DU145 cell monolayers. DU145 cells in confluent monolayers were exposed to 200 μ M KS900 for 4 h (panel A) or 24 h (panel B) under conditions of oxygenation or oxygen deficiency. After treatment, cells were detached by trypsinization and AGT levels were quantified by the [benzene- 3 H]O 6 -benzylguanine binding method. KS900 induced AGT ablation is compared to maximal ablation seen with excess cold O 6 -benzylguanine (BG; 250 μ M). All values are the result of three independent experiments $\pm$ S.E.M.

Fig. 7.

Enhancement of onrigin cytotoxicity by pretreatment with KS900 in DU145 cells. DU145 cells in confluent monolayers were pretreated for 4 h with KS900 (panels A and B), KS90 (panels C and D) or temozolomide (panels E and F) under conditions of oxygenation (panels A, C, E) or oxygen deficiency (panels B, D, F), prior to exposure to incremental amounts of onrigin. Twenty-four hours after the initial treatment, cell toxicity was measured by clonogenic assays. The Y axis indicates the percent survival; the X axis indicates the concentration of onrigin. All points represent at least 3 independent determinations $\pm$ SEM.