Hypoxia-Inducible Factor-1 Inhibition in Combination with Temozolomide Treatment Exhibits Robust Antitumor Efficacy In vivo

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Abstract

Purpose: Inhibiting hypoxia-inducible factor-1 (HIF-1) represents a unique mechanism for cancer therapy. It is conceived that HIF-1 inhibitors may synergize with many classes of cancer therapeutic agents, such as angiogenesis inhibitors and cytotoxic drugs, to achieve a more robust tumor response. However, these hypotheses have not been rigorously tested in tumor models in vivo. The present study was carried out to evaluate the antitumor efficacy of combining HIF-1 inhibition with angiogenesis inhibitors or cytotoxic agents.

Experimental Design: Using a D54MG-derived tumor model that allows knockdown of HIF-1α, we examined the tumor responses to chemotherapeutic agents, including the angiogenesis inhibitor ABT-869 and cytotoxic agents 1,3-bis(2-chloroethyl)-1-nitrosourea and temozolomide, in the presence or absence of an intact HIF-1 pathway.

Results: Surprisingly, inhibiting HIF-1 in tumors treated with the angiogenesis inhibitor ABT-869 did not produce much added benefit compared with ABT-869 treatment alone, suggesting that the combination of an angiogenesis inhibitor with a HIF-1 inhibitor may not be a robust therapeutic regimen. In contrast, the cytotoxic drug temozolomide, when used in combination with HIF-1α knockdown, exhibited a superadditive and likely synergistic therapeutic effect compared with the monotherapy of either treatment alone in the D54MG glioma model.

Conclusions: Our results show that the DNA alkylating agent temozolomide exhibits robust antitumor efficacy when used in combination with HIF-1 inhibition in D54MG-derived tumors, suggesting that the combination of temozolomide with HIF-1 inhibitors might be an effective regimen for cancer therapy. In addition, our results also show that the RNA interference–based inducible knockdown model can be a valuable platform for further evaluation of the combination treatment of other cancer therapeutics with HIF-1 inhibition.

The hypoxia-inducible factor-1 (HIF-1) is a master regulator of cellular responses to low oxygen. It consists of a constitutively expressed β subunit and an oxygen-regulated α subunit. Over the past several years, HIF-1 has emerged as an attractive target for cancer therapy. HIF-1α is expressed in most solid tumors, and high levels of HIF-1α expression are often associated with poor prognosis in cancer patients (1–6). The requirement of HIF-1 for tumor growth has been examined by abrogating the HIF-1 pathway in tumors using genetic means or small-molecule inhibitors. The majority of these studies indicate that inhibition of HIF-1 leads to slower tumor growth in vivo (9–14).

In addition to its direct role on tumor growth, HIF-1 has also been implicated in modulating the tumor response to therapies. A large body of evidence has indicated that hypoxic cancer cells are likely to be more resistant to radiation or cytotoxic drugs, and the drug-resistant phenotype is closely related to the HIF-1 activity in these cells (15–19). Therefore, inhibiting HIF-1 may sensitize hypoxic cancer cells to radiation or cytotoxic drugs and lead to a more profound antitumor efficacy. In addition to radiation and cytotoxic drugs, angiogenesis inhibitors represent another class of anticancer agents that hold promise in combination with HIF-1 inhibitors. It is perceived that antiangiogenesis therapy may enhance tumor hypoxia, and when the hypoxia response is abrogated in cancer cells using HIF-1 inhibitors, a robust antitumor activity may be observed. However, despite the great potential of these combination therapies for cancer, these treatment regimens have not been rigorously tested in tumor models due to technical difficulties.

RNA interference is a process for silencing gene expression using double-stranded RNA (20). Both small interfering RNA and small hairpin RNA (shRNA)–based methods have been used to study the loss-of-function phenotypes of a target protein (21, 22). In our previous studies, we established cancer cell lines that express a shRNA targeting HIF-1α under the tight control of doxycycline and evaluated the therapeutic potential of inhibiting HIF-1 at various stages of tumor development (23). These studies led to the finding that the negative effect of...
inhibiting HIF-1 on tumor growth is transient and tumor stage dependent. The ability of tumors to quickly adapt to the loss of HIF-1 and the resistance of well-established large tumors to HIF-1 inhibition could severely limit the potential applications of HIF-1-based therapy. Here, we describe our effort to overcome these limitations by exploring the possibility of obtaining a robust and sustained tumor response by combining HIF-1 inhibition with other therapeutic agents, including the angiogenesis inhibitor ABT-869 and the cytotoxic drugs temozolomide and carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)].

Materials and Methods

Cell lines, shRNAs, and small interfering RNAs. The D54MG-derived cells expressing a shRNA against luciferase (D54-Luc) or HIF-1α (D54-Hif) on doxycycline induction were established as described previously (23). Cells were maintained in DMEM supplemented with 10% tetrasodium-free fetal bovine serum (Clontech, Palo Alto, CA) in an environment of 5% CO2 at 37°C. For hypoxia treatment, cells were incubated in an environment of 1.5% O2 and 5% CO2 at 37°C. Doxycycline was added to the medium at a final concentration of 1 μg/mL to induce the shRNA expression.

Xenograft models. All cell lines used to generate xenograft tumors were subjected to the IMPACT profile 1 Test (18 agents) at The University of Missouri Research Animal Diagnostic and Investigative Laboratory (Columbia, MO), and each cell line was found negative for all 18 infectious agents tested. Severe combined immunodeficient mice ages 6 to 8 weeks were obtained from the Charles River Laboratories (Wilmington, MA). Cells (5 × 10⁶ per site) were injected s.c. into the hind quarters of mice as a 1:1 mixture with Matrigel (BD Biosciences, San Diego, CA). Tumors were measured twice weekly using a micrometer, and the volume was calculated according to the following formula: (long dimension) × (short dimension)² / 2. Doxycycline (Sigma, St. Louis, MO) was administered through drinking water at 1 mg/mL to induce the expression of shRNA. ABT-869 (Abbott, Abbott Park, IL) was formulated in 2% ethanol, 5% Tween 80, 20% polyethylene glycol (4000), and 0.146% hydroxypropyl methylcellulose for oral administration. Temozolomide (Schering, Kenilworth, NJ) was formulated in 0.2% hydroxypropyl methylcellulose for oral administration. BCNU (carmustine, Bristol, Princeton, NJ) was dissolved in dehydrated ethanol and diluted in D5W for i.p. administration. All animal studies were carried out in accordance with internal Institutional Animal Care and Use Committee guidelines.

Immunohistochemistry. The immunohistochemistry was carried out as described previously (23). Briefly, tumors were excised, cut into pieces <3 mm in thickness, and fixed in Streck tissue fixative (Streck Laboratories, Omaha, NE) solution immediately, and the Streck tissue fixative–fixed and paraffin-embedded tumor sections (5 μm) were used for staining. The mouse anti-HIF-1α monoclonal antibody (Novus, Littleton, CO) was used to detect HIF-1α in tumor samples. The standard H&E procedure was followed to detect necrotic regions. To minimize systemic errors and to avoid the cross-comparison of immunohistochemistry on samples from different experiments or samples processed at different times, immunohistochemistry staining for the same antigen was done on all samples from one experiment as one batch at the same time.

Image acquisition and analysis. Immunohistochemistry images were acquired using the ACSIIS automated imaging system (ChromaVision, San Juan Capistrano, CA). Slides were loaded onto the imaging system, and the machine automatically adjusted the focus and exposure time and captured images of a section using a 10× objective lens by scanning the entire section. Depending on the size of the section, 80 to 400 separate images were acquired to cover the entire section, and these images were automatically composed into one image and exported for analysis. In this study, >200 nonoverlapping images were typically acquired to cover an average section of 50 mm². The images exported from the ACSIIS automated imaging system were analyzed using the “Axiovision 4” software as described previously (23). To ensure an unbiased analysis, the pictures were coded without revealing the identities of the samples to the analyst during the image analysis process. To determine the necrotic index, the necrotic regions were identified based on H&E staining using the criteria of shrinkage or loss of the cell nucleus. The necrotic regions in a section were manually circled, and the software calculated the size of the encircled regions. Tumor necrotic index is calculated using the following formula: size of necrotic regions divided by size of the whole section. To determine the percentage of positive staining areas for HIF-1α, a control slide was used to set the threshold for positive staining and saved as the standard. The same standard was applied for all sections stained against the same antigen in one batch. To exclude necrotic regions from the analysis, the necrotic areas were first manually identified based on the counterstaining. The entire section, excluding the necrotic regions, was selected as the area of interest, and the software automatically calculated the size of positive staining areas in the area of interest and the overall size of the area of interest. The percentage of positive staining area of a marker was calculated using the following formula: size of the positive staining area divided by size of the area of interest (the entire section minus necrotic regions).

Cell growth and cytotoxicity assays. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega, Madison, WI) was used to measure the relative number of viable cells following the manufacturer’s protocol. The ToxiLight assay (Cambrex, Rockland, ME) was used to determine the degrees of cell death according to the manufacturer’s suggestion.

NAD⁺ concentration in cells. The protocol used for the measurement of NAD⁺ concentration was adopted from what was reported in the literature with slight modifications (24). Briefly, 1 × 10⁶ cells were subjected to each treatment as indicated, extracted in 100 μL of 0.5 N perchloric acid, and neutralized with equal volume of neutralization buffer [1 mol/L KOH, 0.33 mol/L phosphate buffer (pH 7.5)]. The cell extract was centrifuged to remove the KCIO₃ precipitates, and 30 μL of the cell extract were added to 120 μL of NAD⁺ reaction mixture [20 μL of MTS/phenazine methosulfate mixture (Promega) plus 100 μL of reaction buffer [720 mmol/L ethanol, 6 mmol/L EDTA, 1.2 mg/mL bovine serum albumin, 144 mmol/L bicarbonate, pH 7.8]]. After 5 minutes of incubation at 37°C, 1.5 μL alcohol dehydrogenase (1,500 units/mL in 50% glycerol; Sigma) was added to the reaction, and the reaction was allowed to proceed for another 20 minutes at 37°C. The reaction was then stopped by the addition of 30 μL of 10% SDS, and the absorbance at 490 nm was measured. The total protein concentration was determined using the RC DC protein assay kit (Bio-Rad, Hercules, CA). The cellular levels of NAD⁺ were determined by comparing the absorbance from the sample and the NAD⁺ standard and then normalized by the total protein concentration.

Statistical analysis and calculation of the enhancement index. The EC50 was calculated by the Prism 4 software (GraphPad Software, San Diego, CA) by fitting data on a sigmoidal dose-response curve using nonlinear regression. The log(EC50) was compared by T-test, and P < 0.05 was considered as significantly different. The enhancement index was used to evaluate the therapeutic synergy between two compounds. It is defined as the median growth delay (treatment index was used to evaluate the therapeutic synergy between two compounds. It is defined as the median growth delay (treatment – control) obtained from the combination therapy divided by the sum of median growth delays (treatment – control) obtained from the monotherapy of each compound. An enhancement index >1 indicates the existence of a therapeutic synergy between the two compounds.

Results

Combining HIF-1 inhibition with the treatment of an angiogenesis inhibitor, ABT-869, did not exhibit a more robust antitumor activity than ABT-869 treatment alone. Antiangiogenesis
therapy is proven to be effective for treating cancers in the clinic. The multitargeted receptor tyrosine kinase inhibitor ABT-869 represents an interesting class of angiogenesis inhibitors that simultaneously target several critical components of the angiogenesis machinery. ABT-869 selectively inhibits the platelet-derived growth factor receptor and KDR family of receptor tyrosine kinases with an ID₅₀ of 4 nmol/L for KDR, 2 nmol/L for FLT1, 14 nmol/L for KIT, 4 nmol/L for FLT3, 190 nmol/L for FLT4, 66 nmol/L for platelet-derived growth factor receptor-β, 3 nmol/L for colony-stimulating factor-1 receptor, and 170 nmol/L for Tie2.

Although ABT-869 does not affect the growth of most cancer cell lines in vitro, including the D54-Hif cells used in this study, it exhibits robust antitumor activities in multiple tumor models (25). It is perceived that the reduction in perfusion induced by an angiogenesis inhibitor, such as ABT-869, could trigger tumor hypoxia, and abrogating the hypoxia response in cancer cells by inhibiting HIF-1 might lead to a more profound antitumor efficacy. To directly test this hypothesis, we created xenograft tumors using the previously described D54-Hif cells, which express a shRNA targeting HIF-1α on exposure to doxycycline. Consistent with what was described in our previous study, HIF-1α knockdown alone had only a moderate effect on tumor growth when the knockdown was initiated at an average tumor size of 190 mm³ (Fig. 1A, doxycycline versus water). ABT-869 treatment alone caused a significant growth inhibition of the D54-Hif-derived tumors (Fig. 1A, ABT-869 versus water). In mice treated with both ABT-869 and doxycycline, tumors slightly regressed from days 36 to 39 (days 6 and 9 after the initiation of therapy), then rebounded to the preregression size at day 45 (day 15 after the initiation of therapy), and continued to grow afterwards (Fig. 1A, ABT-869 plus doxycycline). Despite the transient regression triggered by the combination treatment, combining the HIF-1α knockdown with ABT-869 treatment failed to produce significantly better antitumor efficacy compared with the use of ABT-869 as monotherapy (P = 0.194 for tumor size at day 61, Student’s t test; Fig. 1A, ABT-869 plus doxycycline versus ABT-869, days 33-61). HIF-1α staining revealed an increase of HIF-1α level in tumors from mice treated with ABT-869 for 24 hours (Fig. 1B, left and right, ABT-869 versus control), suggesting that the ABT-869 treatment enhances tumor hypoxia as expected. At this time point, the level of HIF-1α was only slightly reduced in tumors from the doxycycline-treated mice compared with tumors from the untreated mice (Fig. 1B, right, doxycycline versus control). A continued reduction of HIF-1α was observed in tumors from mice treated with doxycycline for 3 and 5 days (Fig. 1C), suggesting that the suboptimal HIF-1α knockdown observed at the 24-hour time point is likely due to the slow induction of shRNA expression in tumors. Although the maximal knockdown of HIF-1α was not reached at the 24-hour time point, doxycycline treatment successfully abrogated ABT-869-induced up-regulation of HIF-1α, suggesting that the hypoxia response induced by ABT-869 treatment is blocked in tumors from mice treated with both ABT-869 and doxycycline (Fig. 1B, left and right, ABT-869 versus ABT-869 plus doxycycline). Taken together, these results suggest that abrogating the tumor hypoxia response triggered by ABT-869 may not provide significant additional benefits for cancer therapy.

It is interesting to note that, in mice that were treated with the combination of ABT-869 and doxycycline, HIF-1α knockdown by itself slowed the tumor growth significantly after ABT-869 withdrawal (Fig. 1A, ABT-869 plus doxycycline versus ABT-869, day 61 and afterwards) despite that the tumors already reached an average size of 370 mm³ at the time of ABT-869 withdrawal. In our previous studies, we have repeatedly observed that the effect of HIF-1α knockdown on tumor growth was closely related to the tumor size. Although the knockdown of HIF-1α caused marked tumor regression and sustained tumor growth delay in small tumors (0-80 mm³), when the D54-Hif-derived tumors reached an average size of 270 mm³, the knockdown of HIF-1α failed to cause any significant growth delay of these tumors (23). Taken together, these results suggest that ABT-869 treatment may partially alleviate the resistance of large tumors to HIF-1α knockdown and cause these tumors to respond to HIF-1 inhibition.

**HIF-1 inhibition in combination with temozolomide treatment exhibited sustained antitumor efficacy.** Cytotoxic drugs, such as the DNA alkylation agents temozolomide and BCNU, are widely used for cancer therapy. To test whether HIF-1 inhibitors could be used in combination with cytotoxic agents to obtain a more robust tumor response, we examined the antitumor efficacy of BCNU or temozolomide when used as a single agent or in combination with HIF-1α knockdown in the D54-Hif-derived tumor xenografts. Mice treated with the combination of BCNU and doxycycline exhibited a similar tumor growth curve compared with mice treated with BCNU alone (P = 0.543 for tumor size at day 48, Student’s t test; Fig. 2, BCNU versus BCNU plus doxycycline, days 29-46). However, similar to what was observed with the combination of ABT-869 and HIF-1α knockdown, in mice that were treated with both BCNU and doxycycline, doxycycline treatment by itself led to a significant delay in tumor growth after BCNU withdrawal (Fig. 2, BCNU versus BCNU plus doxycycline, day 47 and afterwards). Considering that, at the time of BCNU withdrawn (day 47), the tumors already reached an average size of 380 mm³, the slower tumor growth in the doxycycline-treated mice suggests that, like ABT-869, BCNU treatment may also partially alleviate the resistance of well-established large tumors to HIF-1α knockdown and make these tumors to respond to HIF-1 inhibition.

In contrast to what was observed with BCNU, a single dose of temozolomide (7.5 mg/kg at day 33) in combination with the continuous doxycycline treatment (days 33-62) resulted in a transient tumor regression followed by a sustained tumor stasis (Fig. 3A, temozolomide versus temozolomide plus doxycycline), whereas the temozolomide treatment alone only had a moderate effect on tumor growth (Fig. 3A, temozolomide versus water). To determine whether the robust tumor response observed on the combination treatment resulted from a simple additive effect of temozolomide treatment and HIF-1 inhibition, we calculated the enhancement index by dividing the median growth delay (treatment – control) obtained from the combination therapy by the sum of median growth delays (treatment – control) obtained from the monotherapy of each compound. An enhancement index >1 indicates the existence of a superadditive and likely synergistic effect between the two compounds. Enhancement indexes of 1.7, 2.0, and 1.2 were obtained using the combination of temozolomide treatment and HIF-1 inhibition in three independent experiments.
suggesting a therapeutic synergy between temozolomide treatment and HIF-1 inhibition. Consistent with the observed therapeutic synergy between the temozolomide treatment and HIF-1α knockdown, H&E staining of tumor samples revealed that a single dose of temozolomide treatment caused an increase of necrotic regions from posttreatment days 1 to 3. However, the necrotic regions in tumor started to decrease at posttreatment day 5, presumably due to the diminished exposure to temozolomide in these tumors (Fig. 3B, temozolomide). In contrast, in tumors treated with the combination of a single dose of temozolomide plus the continuous exposure of doxycycline, the necrotic regions kept increasing from posttemozolomide treatment days 3 to 5 (Fig. 3B, temozolomide plus doxycycline). Consequently, the degree of tumor necrosis at the post-temozolomide treatment day 5 was markedly higher in tumors excised from mice treated with the combination regimen compared with tumors excised from mice treated with temozolomide or doxycycline alone (Fig. 3B, temozolomide plus doxycycline versus doxycycline or temozolomide at day 5). These results indicate that the combination treatment causes a more severe and sustained damage to the tumor compared with the monotherapy of either temozolomide or doxycycline.

One of the common complications in drug combination studies is the change of pharmacokinetic properties of individual components in the combination regimen due to drug-drug interaction. To exclude the possibility that doxycycline, which is used to induce HIF-1α knockdown, changed the pharmacokinetic properties of temozolomide and led to a superior therapeutic effect, we examined the D54-Luc-derived tumors, which express a shRNA-targeting luciferase on doxycycline induction, for their responses to the treatment of temozolomide as a single agent or in combination with doxycycline. The D54-Luc-derived tumors exhibited overlapping growth curves when treated with temozolomide alone or temozolomide plus doxycycline (Fig. 3C), indicating that doxycycline by itself does not alter the therapeutic effect of temozolomide.

HIF-1α knockdown enhances the cellular activity of temozolomide under hypoxia in a glucose concentration-dependent manner. To understand the mechanism underlying the observed robust antitumor efficacy on the combination of temozolomide treatment and HIF-1α knockdown, we examined the D54-Hif cells in vitro for their responses to the treatment of temozolomide alone or temozolomide in combination with HIF-1α knockdown under hypoxic or normoxic conditions. Our initial experiments failed to detect any differences in cell growth on the treatment of temozolomide alone or temozolomide plus doxycycline under either normoxic or hypoxic conditions, suggesting that our in vitro assay condition may not reflect the in vivo tumor environment (data not shown). Because the regular cell culture medium contains 4.18 g/L glucose, which is much higher than the physiologic glucose concentration, we decided to test whether it is possible to recapitulate the in vivo therapeutic synergy.
cultured in medium containing a physiologic concentration of glucose (1.5 g/L). Knockdown of HIF-1α by itself caused a significant growth inhibition under hypoxic conditions [Fig. 4B, 1.5 g/L glucose versus 1.5 g/L glucose plus doxycycline, log(temozolomide) = -3]. In addition, a 2-fold reduction in the EC$_{50}$ of temozolomide (0.0245 without doxycycline versus 0.0117 with doxycycline; $P = 0.0002$) was observed in hypoxic conditions.
conditions, the HIF-1 knockdown did not cause any growth inhibition or sensitization to temozolomide in cells on HIF-1α knockdown (Fig. 4C, temozolomide EC50, hypoxia, without doxycycline versus with doxycycline), suggesting that knockdown of HIF-1α sensitizes cells to temozolomide treatment under the low glucose and hypoxic conditions. In contrast to what was observed in cells cultured under hypoxia conditions, the HIF-1α knockdown did not cause any growth inhibition or sensitization to temozolomide in cells that were also cultured in medium with the physiologic glucose concentration but under normoxic conditions (Fig. 4A, 1.5 g/L glucose versus 1.5 g/L glucose plus doxycycline; Fig. 4C, temozolomide EC50, normoxia, without doxycycline versus with doxycycline), indicating that the growth inhibition and the sensitization to temozolomide by HIF-1α knockdown depend on both the glucose concentration and the environmental oxygen level. As controls, doxycycline treatment caused neither growth inhibition nor sensitization to temozolomide in the D54-Luc cells under the low glucose and hypoxic conditions (Fig. 5A), indicating that the observed growth inhibition and sensitization to temozolomide in the D54-Hif cells on doxycycline treatment are due to the knockdown of HIF-1α rather than resulting from a nonspecific effect associated with the doxycycline treatment. In addition, consistent with the lack of therapeutic synergy between HIF-1α knockdown and BCNU treatment, doxycycline treatment failed to sensitize the D54-Hif cells to BCNU under the low glucose and hypoxic conditions (Fig. 5B), indicating that the sensitization to temozolomide by HIF-1α knockdown under low glucose and hypoxic conditions is specific for the temozolomide treatment. The growth inhibition by HIF-1α knockdown itself in conjunction with the sensitization of cells to temozolomide treatment under low glucose and hypoxic conditions provide a partial explanation for the observed therapeutic synergy between the temozolomide treatment and HIF-1α knockdown in vivo.

Caspase activation is required for the cell death induced by the combination of temozolomide treatment and HIF-1α knockdown. DNA alkylating agents have been shown to exhibit two
modes of action, the induction of necrotic cell death through a poly(ADP-ribose) polymerase–dependent depletion of cellular NAD⁺ pool and the induction of apoptotic cell death in a caspase-dependent manner (24). It is possible that the knockdown of HIF-1α could enhance the temozolomide-induced NAD⁺ depletion, which will lead to the sensitization of cells to the temozolomide-induced necrotic cell death. To test this hypothesis, we examined the NAD⁺ levels in cells treated with temozolomide alone or temozolomide plus doxycycline. Interestingly, although the temozolomide treatment caused a clear reduction in the cellular NAD⁺ level under normoxic conditions, the temozolomide-induced NAD⁺ depletion was greatly diminished under hypoxic conditions, and combining the HIF-1α knockdown with the temozolomide treatment did not result in a more significant reduction of the cellular NAD⁺ level under hypoxic conditions compared with the temozolomide treatment alone (Fig. 6A). These results suggest that the sensitization of cells to temozolomide on HIF-1α knockdown is unlikely due to the enhancement of temozolomide-induced NAD⁺ depletion by HIF-1α knockdown. Because temozolomide has also been shown to induce apoptotic cell death, we next examined whether the cell death triggered by the combination of temozolomide treatment and HIF-1α knockdown is dependent on caspase activation. Consistent with the observed robust antitumor effect in vivo, the temozolomide treatment combined with HIF-1α knockdown resulted in a more severe cell death compared with temozolomide treatment alone under low glucose and hypoxic conditions (Fig. 6B, without inhibitor, without doxycycline versus with doxycycline). The cell death triggered by the combination treatment of temozolomide and HIF-1α knockdown was completely blocked by caspase inhibitors (Fig. 6B, with inhibitors), suggesting that caspase-dependent apoptosis significantly contributes to the observed cell death on combination treatment of temozolomide and HIF-1α knockdown.

Discussion

Inhibiting HIF-1 represents a unique mechanism for cancer therapy. It is conceived that HIF-1 inhibitors may synergize with many classes of cancer therapeutic agents, such as angiogenesis inhibitors and cytotoxic drugs, to achieve a more robust tumor response. However, these hypotheses have not been rigorously tested in tumors. The RNA interference–based inducible knockdown system, with its flexibility and specificity, can be a valuable tool for evaluating cancer targets in vivo. Using a D54MG-derived tumor model that allows knockdown of HIF-1α in vivo on doxycycline treatment, we examined the tumor responses to various chemotherapeutic agents in the presence or absence of an intact HIF-1 pathway. Surprisingly, inhibiting HIF-1 in tumors treated with the angiogenesis inhibitor ABT-869 did not produce much added benefit compared with ABT-869 treatment alone, suggesting that abrogating the tumor hypoxia response triggered by ABT-869 treatment may not provide significant additional benefits for cancer therapy.

In contrast, the cytotoxic drug temozolomide, when used in combination with HIF-1α knockdown, exhibited a super additive and likely synergistic therapeutic effect compared with the monotherapy of either treatment alone in the D54MG glioma model. The persistent tumor stasis in response to the combination treatment suggests that HIF-1 inhibitors, when used in combination with temozolomide, could have clinical benefit by effectively blocking tumor progression between two cycles of temozolomide treatment.

The observed robust antitumor efficacy on the combination of temozolomide treatment and HIF-1α knockdown could be partially explained by the growth inhibition triggered by HIF-1α knockdown itself in conjunction with the sensitization of cells to temozolomide treatment by HIF-1α knockdown in low glucose and hypoxic conditions. However, considering that temozolomide is only dosed once at day 1, the above explanations may not fully account for the persistent tumor stasis observed on the combination of temozolomide treatment and HIF-1 inhibition. We have shown previously that medium-sized tumors (average size of 190 mm³) can quickly adapt to the loss of HIF-1 and continue to grow (23). These tumors often exhibit a very transient stasis followed by quick progression on the inhibition of HIF-1. The persistent tumor stasis observed on the combination treatment of temozolomide and HIF-1α knockdown suggests that the temozolomide treatment might damage the tumor adaptation response and cause a delay in tumor adaptation to the loss of HIF-1.
It is interesting to note that both ABT-869 and BCNU treatments caused large tumors to respond to HIF-1α knockdown, although these tumors are resistant to HIF-1 inhibition without the preexposure to ABT-869 or BCNU (23). We speculate that both ABT-869 and BCNU treatments might also hit some components that are responsible for the resistance of large tumors to HIF-1 inhibition and therefore cause a partial reversal of resistance to HIF-1 inhibition in large tumors. However, a mechanistic explanation for how temozolomide, BCNU, and ABT-869 might alleviate the tumor adaptation/resistance to HIF-1 inhibition will first require a clear understanding of how tumors become adapted/resistant to HIF-1 inhibition.

The glucose-dependent effect of HIF-1α knockdown on cell growth is of great interest. We have shown that the loss of HIF-1α does not directly affect tumor cell growth under both normoxic and hypoxic conditions (23). The disconnection between the in vitro cell growth and the in vivo tumor growth suggests that the in vivo efficacy of HIF-1 inhibition might be due to an indirect effect, such as the inhibition of angiogenesis. However, results from others and us show that inhibiting HIF-1 in tumors only has marginal effect on the tumor vessel density (14, 23). The observed direct effect of HIF-1α knockdown on cell growth under the physiologic glucose concentration helps to resolve the paradox by supporting the hypothesis that, in our D54MG-derived tumor models, the in vivo efficacy of HIF-1 inhibition might primarily come from a direct inhibition of tumor cell growth rather than from an indirect effect on tumor angiogenesis. In addition, the glucose-dependent effect of HIF-1α knockdown on cell growth also provides a potential explanation for the tumor adaptation to the loss of HIF-1. We have previously shown that the transient tumor stasis in response to HIF-1α knockdown seemed to track the change of Glut1 levels in tumors, and a clear increase of Glut1 staining has been observed before the tumors exit stasis and regain growth (23). It is conceivable that an increase of Glut1 may lead to a more efficient transport of glucose into tumor cells and boost the cellular glucose concentration to a level that makes the cells no longer respond to HIF-1 inhibition.

In summary, using the previously described inducible knockdown tumor model, we examined the combination of several cancer therapeutic agents with HIF-1 inhibition for their antitumor efficacy. Our results show that the DNA alkylating agent temozolomide exhibits robust antitumor efficacy when used in combination with HIF-1 inhibition in D54MG-derived tumors, suggesting that the combination of temozolomide with HIF-1 inhibitors might be an effective regimen for cancer therapy. In addition, our results also show that the RNA interference–based inducible knockdown model can be a valuable platform for further evaluation of the combination treatment of other cancer therapeutics with HIF-1 inhibition.

References
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