Reversing Hypoxic Cell Chemoresistance in Vitro Using Genetic and Small Molecule Approaches Targeting Hypoxia Inducible Factor-1

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ABSTRACT

The resistance of hypoxic cells to conventional chemotherapy is well documented. Using both adenovirus-mediated gene delivery and small molecules targeting hypoxia-inducible factor-1 (HIF-1), we evaluated the impact of HIF-1 inhibition on the sensitivity of hypoxic tumor cells to etoposide. The genetic therapy exploited a truncated HIF-1α protein that acts as a dominant-negative HIF-1α (HIF-1α-no-TAD). Its functionality was validated in six human tumor cell lines using HIF-1 reporter assays. An EGFP-fused protein demonstrated that the dominant-negative HIF-1α was nucleus-localized and constitutively expressed irrespective of oxygen tension. The small molecules studied were quinocarmycin monocitrate (KW2152), its analog 7-cyanoquinocarcinol (DX-52-1), and topotecan. DX-52-1 and topotecan have been previously established as HIF-1 inhibitors. HT1080 and HCT116 cells were treated with either AdHIF-1α-no-TAD or nontoxic concentrations (0.1 μM; $<IC_{10}$) of KW2152 and DX-52-1 and exposed to etoposide in air or anoxia ($<0.01\%$ oxygen). Topotecan inhibited HIF-1 activity only at cytotoxic concentrations and was not used in the combination study. Etoposide IC_{50} values in anoxia were 3-fold higher than those in air for HT1080 (2.2 $\pm$ 0.3 versus 0.7 $\pm$ 0.2 μM) and HCT116 (9 $\pm$ 4 versus 3 $\pm$ 2 μM) cells. KW2152 and DX-52-1 significantly reduced the anoxic etoposide IC_{50} in HT1080 cells, whereas only KW2152 yielded sensitization in HCT116 cells. In contrast, AdHIF-1α-no-TAD (multiplicity of infection 50) ablated the anoxic resistance in both cell lines (IC_{50} values: HT1080, 0.7 $\pm$ 0.04 μM; HCT116, 3 $\pm$ 1 μM). HIF-1α-no-TAD expression inhibited HIF-1-mediated down-regulation of the proapoptotic protein Bid under anoxia. These data support the potential development of HIF-1 targeted approaches in combination with chemotherapy, where hypoxic cell resistance contributes to treatment failure.

The resistance of hypoxic cells to anticancer therapeutic strategies has a profound impact on treatment response. Within solid tumors, hypoxic cell chemoresistance was originally attributed to poor drug distributions and to the contention that hypoxic tumor cells are predominantly quiescent. Preclinical observations have additionally demonstrated that hypoxia is a selective pressure for genomic instability with acquired drug resistance and loss of apoptotic potential reported for cells exposed to hypoxia both in vitro and in vivo (Young et al., 1988; Teicher, 1994; Graeber et al., 1996).

More recently a contributory role for the transcription factor hypoxia-inducible factor-1 (HIF-1) has been revealed (Comerford et al., 2002; Unruh et al., 2003; Erler et al., 2004). HIF-1, a heterodimer composed of HIF-1α and HIF-1β subunits, is a pivotal regulator of gene transcription in response to hypoxia (Wiesener and Maxwell, 2003). The HIF-1α subunit is oxygen-labile and is subject to ubiquitination and proteosomal degradation under aerobic conditions. The enzymes responsible for the post-translational modification of HIF-1α that targets the protein for degradation cannot function at low oxygen tension. This enables accumulation of

ABBREVIATIONS: HIF-1, hypoxia-inducible factor-1; TAD, transactivation domain; EF-1, elongation factor-1α; m.o.i., multiplicities of infection; HRE, hypoxia-responsive element; LDH, lactate dehydrogenase; wt, wild type; EGFP, enhanced green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; KW2152, 2a,3,4,5,6,6a,7,11b-octahydro-11-methoxy-12-methyl-3,6-imino-1H-2-oxa-11c-azanaphth(1,2,3-cd)azulene-5-carboxylic acid monocitrate; quinocarmycin citrate; DX-52-1, 7-cyanoquinocarcinol (NSC-607097); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PGK, phosphoglycerate kinase; ODDD, oxygen-dependent degradation domain; NLS, nuclear localization signal.

HIF-1α and formation of the HIF-1 complex under hypoxic conditions (Wiesener and Maxwell, 2003; Metzen and Ratcliffe, 2004). The differential gene expression pattern achieved as a consequence of HIF-1 activation promotes a survival advantage in low oxygen conditions, and xenograft studies using HIF-1 deficient models have generally established HIF-1 as a positive factor in tumor growth (Jiang et al., 1997; Maxwell et al., 1997; Ryan et al., 1998; Griffiths et al., 2002; Williams et al., 2002).

These data have contributed to the proposal that HIF-1 is a potential target for therapeutic intervention. Further support comes for the observations that HIF-1 can be inappropriately activated in malignant disease through both oncogene activation and tumor suppressor loss (Bardos and Ashcroft, 2004). HIF-1 expression has been consistently recorded in the vast majority of solid human tumors and has shown positive correlations with both advancing tumor grade and poorer response to therapy (Zhong et al., 1999; Birner et al., 2000; Bos et al., 2001).

The recent studies highlighting the impact of HIF-1 on chemotherapy response add an additional dimension to the use of HIF-1 inhibitors as potential enhancers of standard chemotherapeutics. Unruh et al. (2003) reported that transformed mouse embryonic fibroblasts lacking HIF-1α were more chemoresponsive to carboplatin and etoposide than wild-type mouse embryonic fibroblasts. In this study, we sought to establish whether HIF-1 targeting using a genetic approach or small molecules is a valid strategy to sensitize human tumor cells to chemotherapy and, in particular, to reverse hypoxic cell chemoresistance. The genetic approach used a truncated, dominant-negative variant of HIF-1α expressed in an adenoviral context. The small molecules used were quinocarmycin monocitrate (KW2152), its hydrocyanation product DX-52-1, and the topoisomerase I inhibitor topotecan. The latter two agents were revealed as HIF-1 inhibitors upon screening of the National Cancer Institute diversity set (Rapisarda et al., 2002). We investigated the impact of the dominant-negative HIF-1α and small molecules on the chemotherapeutic response of tumor cells in vitro. We used etoposide as a model anticancer agent because we have shown previously that oxygen deprivation results in etoposide resistance (Erler et al., 2004).

Materials and Methods

Vector Construction. The method for generating pcDNA3.1/Zeo expressing a truncated HIF-1α construct lacking the transactivation domains (TAD) was based on a published strategy (Jiang et al., 1996). The TAD was removed from the HIF-1α cDNA (contained in pBSSKII+) using NotI/AflII, and a linker was inserted (5′-TTAAGT-GAGCTTTTTCTTAATCTAGAGC-3′). HIF-1α-no-TAD (1.1-kilobase fragment) was then isolated (NotI/KpnI) and cloned into the pcDNA3.1/Zeo vector (Invitrogen, Paisley, UK) generating pHIF-1α-no-TAD. To construct pHIF-1α-no-TAD-EGFP, the sequence encoding EGFP was removed from pIRES2EGFP (Clontech, Mountain View, CA) and introduced upstream and in frame of HIF-1α-no-TAD in pBSSKII+. HIF-1α-no-TAD-EGFP was isolated by NotI/KpnI digestion and cloned into pcDNA3.1/Zeo to generate pHIF-1α-no-TAD-EGFP.

Adenoviral Vectors. AdHIF-1α-no-TAD was made using the pAd Easy system (Stratagene, La Jolla, CA). HIF-1α-no-TAD was isolated and cloned into a modified pShuttle containing the elongation factor-1α (EF-1) promoter. pShuttle-HIF-1α-no-TAD and pAdEasy were cotransformed into BJ5183 competent cells (Strat-

agene, Cambridge, UK) generating pAdHIF-1α-no-TAD. Primary viral inocula and large-scale preparations were generated in human embryonic kidney 293 cells (Cowen et al., 2004) and purified using the Adeno-X Virus Purification kit (Clontech). In the experimental studies, cells were infected with either Adβ-galactosidase or AdHIF-1α-no-TAD at increasing multiplicities of infection (m.o.i.; number of infectious viral particles per cell) 48 h before use.

HIF-1 Reporter Constructs. Reporter vectors encompassed trimers of the hypoxia-responsive elements (HRE) isolated from the *LDH-A* (GCGGACGTGCGGGAACCCACGTGTA) and *PGK-1* (TGTCACGTCCT GCACGACGCGAGTA) genes. These were cloned 5′ to the simian virus 40 minimal promoter sequence upstream of firefly luc^+ in the pGL3-promoter vector (Promega, Southampton, UK). The lactate dehydrogenase (LDH) sequence was cloned in the reverse orientation with respect to the promoter. The carbonic anhydrase-9 reporter has been described previously (Wykoff et al., 2000) and consisted of the sequence −506/+43 in the native gene cloned into pGL3-basic (Promega).

Cell Lines. All of the human tumor cell lines [which included HT1080 (fibrosarcoma), DU145 (prostate carcinoma), U87 (glioma), T47D (breast carcinoma), and HCT116 and HT29 (colon carcinoma)] and the rodent lines were cultured in RPMI medium (Invitrogen) containing 10% fetal calf serum and 2 mM glutamine in a 95% air/5% CO_2 environment. All were free from mycoplasma contamination (Mycotect; Invitrogen). The Chinese hamster ovary-K1 derived HRE-reporter strain, C4.5, expresses human CD-2 regulated by the murine PGK-1-HRE sequence. Ka13.5 cells were derived from C4.5 and are HIF-1α-deficient (Wood et al., 1998). The HCT116 HRE-reporter strain expresses the LDH-A simian virus 40 minimal promoter sequence-driven Firefly $luc+$ cassette detailed above encoded within pCI-neo (Promega). Exposure to anoxia or the hypoxic mimetic cobalt chloride (100 μM) for 16 h yields a robust (~10-fold) induction of luciferase activity in these cells (K. J. Williams and R. L. Cowen, unpublished observations). The Hepa-1 wt and HIF-1β-deficient derivative Hepa-1 c4 are murine hepatoma cells and have been described extensively (Maxwell et al., 1997; Ryan et al., 1998; Griffiths et al., 2002; Williams et al., 2002).

Transient Transfections. Transfections were performed on exponential phase cells using Lipofectamine (Invitrogen) and 1 μg of DNA per 2 × 10^5 cells. For HRE-luciferase reporter experiments, cells were transfected with equal amounts of reporter plasmid and either pHIF-1α-no-TAD or blank DNA (pGL3-basic). For analysis of effects on constitutive gene expression, cells were transfected with a *Renilla reniformis* luciferase reporter vector, in which *R. reniformis* $luc+$ was cloned upstream of the human EF-1 promoter. On the following day, transfected cells were subcultured into replicate plates and subjected to 16 h of an aerobic or anoxic environment (5% CO_2/5% H_2/90% N_2 passed over a palladium catalyst to remove residual oxygen; oxygen concentration <0.01%; Bactron anaerobic chamber; Sheldon Manufacturing, Cornelius, OR). Luciferase activity was determined using a manufactured kit (Promega). To analyze EGFP fluorescence after transfection with pIRES2EGFP or pHIF-1α-no-TAD-EGFP, cells were cultured on sterile glass coverslips. Nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) and relative localization of green (EGFP) and blue (DAPI) fluorescence analyzed using an Axioplan microscope (Zeiss, Jena, Germany) and associated software.

Western Blot Analysis. For EGFP protein detection, transfected cells were lysed using the Nuclei EZ Prep Nuclei Isolation kit (Sigma Chemical, Poole, Dorset, UK), and nuclear and cytoplasmic samples were prepared. Aliquots containing 40 μg of protein were run on 10% SDS polyacrylamide gels and then transferred to Hybond membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). EGFP-containing proteins were detected using mouse anti-EGFP JL-8 antibody (1:1000; Clontech) followed by anti-mouse horseradish peroxidase-conjugated IgG (1:2500; Sigma); enhanced chemiluminescence (GE Healthcare) was used to identify relevant bands. For Bid protein detection, whole-cell lysates were prepared and 20 μg of

protein was resolved using 15% gels. Bid was detected using 1:1 mixture of 1:500 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and 1:2000 antibodies from R&D Systems Europe Ltd. (Abingdon, Oxfordshire, UK), both raised in goat (Erler et al., 2004). Actin was revealed using AC40 antibody (1:2000; Sigma).

Carbonic Anhydrase Activity Assay. Carbonic anhydrase activity was determined as a surrogate marker for HIF-1 function (Wykoff et al., 2000) in whole-cell lysates prepared in hypotonic buffer supplemented with protease inhibitors (10 mM HEPES, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin) using the Wilbur-Anderson method (Wilbur and Anderson, 1984). The time required (seconds) for a saturated CO_2 solution to lower the pH of 0.02 M Tris-HCl from 8.3 to 6.3 was determined as a marker for carbonic anhydrase activity. Assays were performed on ice using 0.01 mg/ml lyophilized carbonic anhydrase (Worthington Biochemicals, Freehold, NJ) as a positive control. Time without enzyme (T_0) was determined by placing 6 ml of 0.02 M Tris-HCl on ice and recording the pH. Four milliliters of ice-cold CO_2-saturated water was added, and the time taken for the 2-unit pH change was recorded. Time with enzyme (T) was ascertained by adding 100 μl of the positive control or sample. Carbonic anhydrase activity (units per milligram) was determined as $2 \times (T_0 - T)/T \times$ milligrams of protein.

Cytotoxicity Studies. Cells (untreated or virally infected) were subcultured into 96-well plates (2500 cells per well). For anoxic exposure, cells were cultured within the anoxic chamber using primed medium and plastics. After allowing for cell attachment, etoposide (Sigma), KW2152, DX-52-1 [National Cancer Institute, Bethesda, MD (Rapisarda et al., 2002)], or topotecan [GlaxoSmith-Kline (Welwyn Garden City, Hertfordshire, UK)/Merck Sharp and Dohme (Hoddesdon, UK)] were added at a range of concentrations for 16 h. Drug containing medium was replaced with fresh medium, and cells were cultured for 3 days under standard (aerobic) conditions. The influence of drug exposure on cell proliferation was ascertained by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as described previously (Cowen et al., 2004). In the combination studies, KW2152 and DX-52-1 were added 1 h before and maintained throughout the period of etoposide exposure. Preliminary validation studies were undertaken to ensure that the duration of anoxia used did not significantly compromise proliferation and viability.

Cell Cycle Analysis. Exponentially growing cells were seeded into six-well plates at a concentration of 1.5×10^6 cells per well. On the following day, the cells were exposed to anoxia or cultured under standard aerobic conditions for 16 h. Cells were fixed, treated with RNase-A, stained with propidium iodide, and analyzed by flow cytometry (FACSort; BD Biosciences, San Jose, CA) using standard techniques (Williams et al., 1996).

Results

Subcellular Localization and Oxygen Dependence of HIF-1α-no-TAD. To generate the HIF-1 dominant-negative, HIF-1α-no-TAD, the TAD were removed from the coding sequence of human HIF-1α. This deleted the oxygen-dependent degradation domain (ODDD) but left those regions required for dimerization and DNA binding intact (Fig. 1A). To ease investigation of the subcellular localization of the dominant-negative HIF-1α, the coding sequence for EGFP was fused in frame to the C terminus of HIF-1α-no-TAD. Cells were transfected with pHIF-1α-no-TAD-EGFP and subjected to 16 h of air or anoxia. HIF-1α-no-TAD-EGFP localized to the nucleus and was expressed equivalently in aerobic and anoxic conditions (Fig. 1B). Transfection with the control vector pIRESEGFP resulted in EGFP expression that was

confined to the cytoplasm (Fig. 1B). These findings were confirmed by Western blot analysis for EGFP, where the 85-kDa EGFP-fused HIF-1α-no-TAD was clearly evident in only the nuclear preparation (Fig. 1C).

Functionality of HIF-1α-no-TAD. The ability of HIF-1α-no-TAD to interfere with HIF-1-mediated gene expression was evaluated against a luciferase reporter construct comprising a trimer of the HIF-1 recognition sequence (HRE) from the LDH promoter. A range of tumor cell lines was transiently transfected with the LDH-HRE reporter construct alone or in combination with pHIF-1α-no-TAD. The dominant-negative HIF-1α decreased HRE-mediated luciferase expression in all cell lines in anoxia (Fig. 1D). Significant down-regulation of aerobic reporter expression was achieved in HT1080, DU145, and U87 cells (Fig. 1D; Table 1). Further evaluation was undertaken using T47D and HT1080 cells and demonstrated that HIF-1α-no-TAD could similarly inhibit expression driven by the HREs from the phosphoglycerate kinase (PGK) and carbonic anhydrase-9 genes in both air and anoxia (Table 1). In contrast with the strong inhibitory effect of HIF-1α-no-TAD on expression mediated through HRE elements in a minimal simian virus 40 promoter context, HIF-1α-no-TAD had no significant effect on gene expression regulated by the constitutive human EF-1 promoter in the cell line panel (data not shown).

Validation of an Adenoviral Vector Expressing HIF-1α-no-TAD. HIF-1α-no-TAD was cloned into a replication-deficient type 5 adenovirus vector to generate AdHIF-1α-no-TAD. Adenoviral infection of HCT116 cells that stably express the LDH-HRE-luciferase cassette (Fig. 2A) inhibited reporter induction after anoxic exposure. Similar results were obtained in C4.5 cells that stably express the CD-2 cell surface marker regulated by a PGK-HRE sequence, whereas neither control virus (Adβ-galactosidase) nor AdHIF-1α-no-TAD had any effect on reporter expression in the C4.5 derived Ka13.5 cells that lack HIF-1 function (data not shown). To elucidate the effect of AdHIF-1α-no-TAD on endogenous HIF-1-mediated gene expression a carbonic anhydrase-9 activity assay was performed after anoxic or aerobic exposure of virally infected cells. HT29 cells were used as they exhibit the highest anoxic induction of carbonic anhydrase-9 of the cell line panel (Williams et al., 2005). Carbonic anhydrase-9 activity in HT29 cells treated with control virus (50 viral particles per cell; m.o.i. 50) was 1.05 ± 0.07 units/mg in air and 2.64 ± 0.50 units/mg after anoxic exposure. AdHIF-1α-no-TAD treatment (m.o.i. 50) inhibited the activity of carbonic anhydrase-9 by 6-fold in air and resulted in almost undetectable levels of carbonic anhydrase-9 activity in anoxic conditions (0.22 ± 0.05 and 0.02 ± 0.01 units/mg, respectively).

Targeting HIF-1 Transactivation Using Small Molecules. Consistent with previous observations using stably transfected U251 human glioma cells (Rapisarda et al., 2002), KW2152, DX-52-1, and topotecan caused a concentration-dependent inhibition of HRE-mediated expression in the HCT116 reporter line (Fig. 2B, and data not shown). The concentration of DX-52-1 or KW2152 required to yield a 50% inhibition of HRE-mediated expression (300 nM) was below the concentration causing a 50% reduction in proliferation (IC_{50}) in HCT116 wild-type cells (1.2 ± 0.4 and 0.7 ± 0.3 μM for DX-52-1 and KW2152, respectively). This was not the case for topotecan, where 50% HIF-1 inhibition was only achieved using a concentration 12-fold higher (600 nM) than

the IC_{50} (0.05 ± 0.04 μM). Furthermore, the concentration of topotecan required to inhibit constitutive, EF-1-driven reporter expression by 50% was lower than that required for HRE inhibition (100 nM). This was not the case for DX-52-1 and KW2152 with 50% inhibition of EF-1-mediated expression achieved using concentrations of 900 and 800 nM, respectively.

AdHIF-1α-no-TAD and Nontoxic Doses of DX-52-1 and KW2152 Can Reverse the Anoxic Resistance of Tumor Cells to Etoposide. Preliminary studies were undertaken using Hepa-1 wt and the HIF-1β-deficient Hepa-1 c4 to establish that HIF-1 function affects etoposide sensitivity. A 2-fold increase in etoposide concentration was required to give the same level of cell kill in anoxic conditions com-

pared with aerobic exposure in the Hepa-1 wt cells. The HIF-1-deficient Hepa-1 c4 cells, however, were more sensitive to etoposide in anoxia (Table 2). This was not attributable to any effects of anoxia on cell cycle distribution, which was not significantly changed compared with cells cultured in aerobic conditions (Table 3). Both HT1080 and HCT116 cells show an inherent resistance to etoposide under anoxic conditions. Again the anoxic drug resistance could not be related to cell cycle characteristics that were unaffected by the 16-h anoxic exposure used (Table 3). The IC_{50} values after a 16-h exposure to etoposide under aerobic or anoxic conditions were 0.7 ± 0.2 and 2.2 ± 0.3 μM for HT1080 cells and 3 ± 2 and 9 ± 4 μM for HCT116 cells. Pretreatment with AdHIF-1α-no-TAD decreased the anoxic etoposide IC_{50} con-
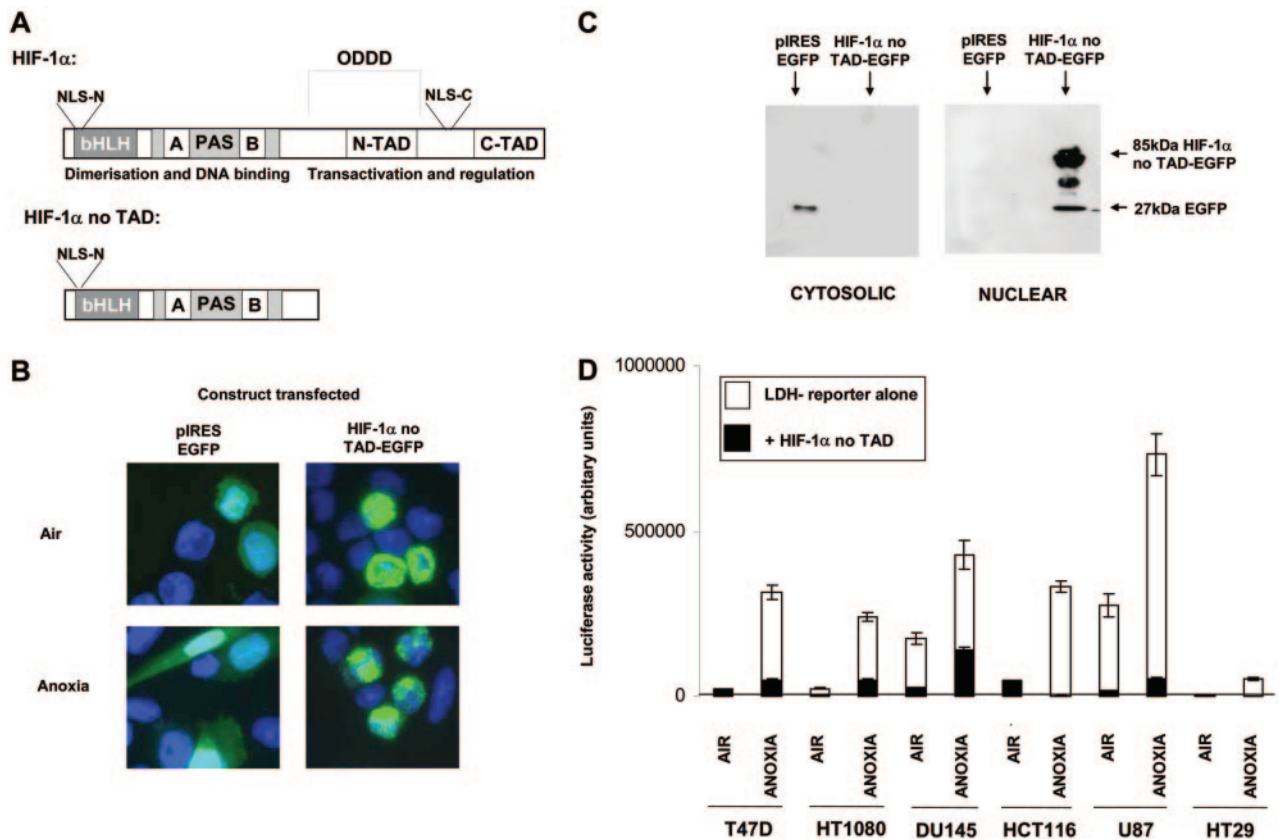
Fig. 1. A, schematic of HIF-1α and the truncated, dominant-negative HIF-1α-no-TAD. B, HIF-1α-no-TAD is nuclear localized and expressed independently of oxygen availability. C4.5 cells were transfected with pIRESEGFP or pHIF-1α-no-TAD-EGFP, and EGFP was visualized using fluorescence microscopy. EGFP colocalizes with nuclear DAPI staining in pHIF-1α-no-TAD-EGFP–expressing cells, whereas EGFP encoded by pIRESEGFP is cytoplasmic. C, nuclear localization of the pHIF-1α-no-TAD-EGFP construct was confirmed by Western blotting cytosolic and nuclear extracts of transfected cells using an antibody raised against EGFP. D, HIF-1α-no-TAD acts as a dominant negative of HIF-1 function. Human tumor cells were cotransfected with LDH-HRE-luciferase and either pHIF-1α-no-TAD (closed bars) or blank DNA (open bars) and exposed to aerobic or anoxic conditions for 16 h, and luciferase activity was determined. Data shown are average values $\pm$ S.E. ($n = 3$).

TABLE 1

Inhibitory effect of plasmid driven HIF-1-α-no-TAD expression on HRE reporter output

-Fold inhibition was calculated by dividing the luciferase activity obtained upon transfection of reporter alone with the activity obtained when co-transfected with HIF-1α-no-TAD.

Cell Line	Tumor Type	LDH		PGK		CA-9		VEGF	
		Air	Anoxia	Air	Anoxia	Air	Anoxia	Air	Anoxia
T47D	Breast	0.3	6.5	0.8	5.2	0.8	5.4	1.3	0.5
HT1080	Fibrosarcoma	4.7	5.2	1.7	6.0	2.3	5.4	0.4	1.5
DU145	Prostate	7.1	3.0						
HCT116	Colon	0.4	181.5						
U87	Glioma	16.2	14.3						
HT29	Colon	1.5	33.7						

centration. An m.o.i. of 50 was sufficient to sensitize both HT1080 and HCT116 cells to achieve IC_{50} concentrations that mimicked those in air (Fig. 3, A and B, Table 2, and data not shown). Relative resistance values (anoxic IC_{50}/aerobic IC_{50}) were calculated from three independent experiments
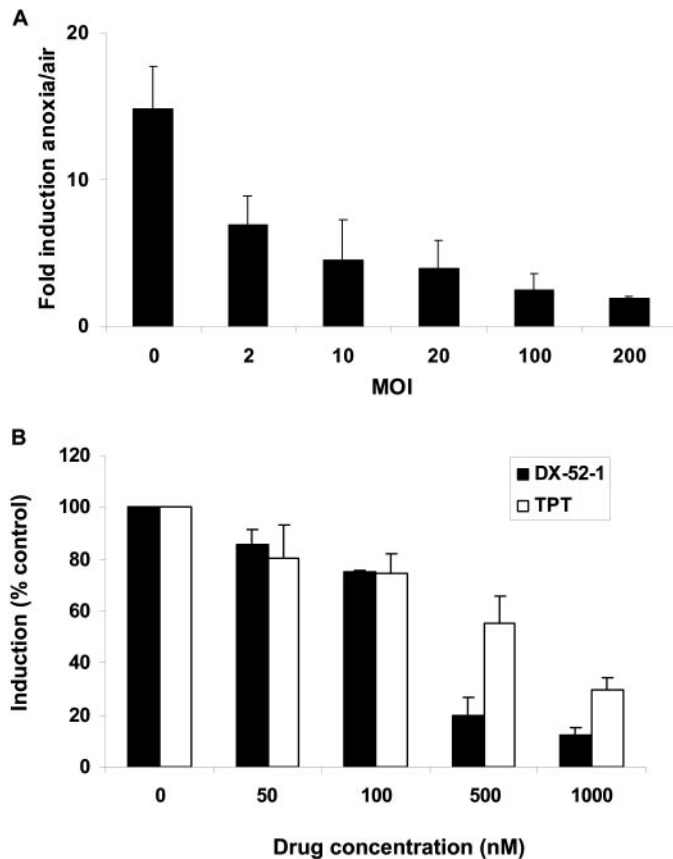

Fig. 2. A, AdHIF-1α-no-TAD inhibits anoxia-mediated induction of HRE-reporter expression in HCT116 cells with a stably integrated HRE-reporter construct. Cells were infected with increasing infectious viral particles per cell (m.o.i.) 48 h before exposure of cells to air or anoxia for 16 h. The data are averages of reporter induction $\pm$ S.E. ($n = 3$). B, topotecan (TPT) and DX-52-1 inhibit 100 μM cobalt chloride-mediated induction of luciferase in HCT116 HRE-reporter cells. Drugs were added 1 h before and maintained throughout the 16-h exposure to the hypoxic mimetic cobalt chloride. Data presented were determined from three experiments ($\pm$ S.E.).

and show that at an m.o.i. of 50, there was some aerobic etoposide sensitization in the HT1080 but not HCT116 cells (Table 2). Pretreatment with control virus had no effect (Fig. 3; Table 2). A nontoxic drug concentration was determined for the small molecule inhibitors that caused less than 10% growth inhibition in HT1080 and HCT116 cells. The concentration used was 100 nM. In addition to causing minimal growth effects, this concentration had no effect on constitutive, EF-1-driven gene expression under either aerobic or anoxic conditions (data not shown). DX-52-1 and KW2152 were dosed at 100 nM in combination with etoposide treatment. The direct cytotoxicity of topotecan precluded its use in the combination studies. In HT1080 cells, cotreatment with either drug significantly reduced the IC_{50} for etoposide when exposed under anoxic conditions but had no significant effect on the aerobic IC_{50} value (Fig. 3C). KW2152 cotreatment afforded some reduction in the relative resistance of HCT116 cells to etoposide treatment under anoxic conditions, whereas DX-52-1 had no effect (Table 2).

We have recently elucidated the proapoptotic protein Bid as a target for HIF-1-mediated down-regulation under anoxic conditions (Erler et al., 2004). Therefore, we evaluated the impact of the dominant-negative HIF-1α and DX-52-1 on the expression of Bid. HIF-1α-no-TAD consistently reversed the anoxic down-regulation of Bid observed in untreated or empty vector control samples of both HT1080 and HCT116 cells (Fig. 3D). It is noteworthy that this was also apparent using 100 nM DX-52-1, although chemosensitization was seen only in HT1080 cells (Fig. 3D).

Discussion

HIF-1 has emerged as a target for the development of anticancer therapeutics. This was initially underpinned by xenograft studies that established a pro-tumor role for HIF-1 (Maxwell et al., 1997; Ryan et al., 1998; Griffiths et al., 2002; Williams et al., 2002) and supported targeted disruption of HIF-1 transactivation as a strategy to reduce tumor growth (Kung et al., 2000; Chen et al., 2003; Stoeltzing et al., 2004). Compelling supportive data have arisen through the observations that HIF-1 has an influential role in modulating chemotherapeutic response (Unruh et al., 2003; Erler et al., 2004). If approaches targeting HIF-1 could be rationally applied in the context of standard chemotherapy, there is po-

TABLE 2
The effect of HIF-1 targeting on the sensitivity of tumor cells to exposure to etoposide for 16 h under aerobic and anoxic conditions

To gain resistance factor values for HT1080 and HCT116 cells, IC_{50} values obtained under each experimental condition were related to the aerobic IC_{50} value. For the Hepa-1 cells, a clonogenic assay was used, and the drug dose required to give 1% survival under anoxia was related to that required to give the same level of survival in air. Data presented are mean values $\pm$ S.E.M.

Cell Line	Pre- or Cotreatment	Resistance Factor	
		Air	Anoxia
Hepa-1 wt	None	1	1.98 ± 0.08
Hepa-1 c4[a]	None	1	0.52 ± 0.08
HT1080	None	1	3.06 ± 0.37
	Control virus m.o.i. 50	0.88 ± 0.30	4.69 ± 1.45
	Ad HIF-1α-no-TAD m.o.i. 50	0.56 ± 0.17	0.96 ± 0.06
	KW2152 0.1 μM	0.91 ± 0.37	0.78 ± 0.11
	DX-52–1 0.1 μM	1.02 ± 0.51	1.31 ± 0.31
HCT116	None	1	1.91 ± 0.22
	Control virus m.o.i. 50	1.01 ± 0.04	2.06 ± 0.21
	Ad HIF-1α-no-TAD m.o.i. 50	1.17 ± 0.02	0.74 ± 0.34
	KW2152 0.1 μM	0.62 ± 0.07	1.42 ± 0.09
	DX-52–1 0.1 μM	0.54 ± 0.05	1.92 ± 0.62

[a] Hepa-1 c4 cells lack HIF-1 function through a deficiency in HIF-1β.

tential for a greater therapeutic efficacy than could be achieved through targeting HIF-1 alone. This is particularly true in light of the results of studies using admixed populations of HIF-1 wild-type and -deficient cells demonstrating that a 1:100 concentration of wild-type cells is sufficient to rescue the in vivo growth of an HIF-1-deficient population (Hopfl et al., 2002). In this study, we have provided proof of principle data that support the application of HIF-1-targeted approaches in combination with etoposide chemotherapy to reverse hypoxia-mediated drug resistance.

We have evaluated both the use of a truncated HIF-1α protein (HIF-1α-no-TAD) that acts as a dominant-negative inhibitor of HIF-1 function and small molecule inhibitors (the camptothecin analog topotecan and DX-52-1, the hydrocyanation product of KW2152) that were revealed as potential HIF-1 inhibitors in the study of Rapisarda (2002). This pilot study in which the NCI diversity set of 2000 compounds was

evaluated reported four hits (topotecan, two further campto-thecin analogs, and DX-52-1) that could inhibit both HRE-reporter and endogenous HIF-1-dependent (vascular endo-thelial growth factor) expression. Subsequently a number of additional small molecule inhibitors of HIF-1 function have been identified (Mabjeesh et al., 2003; Yeo et al., 2003; Welsh et al., 2004; Tan et al., 2005).

The generation of the dominant-negative HIF-1α involved deleting the N- and C-TAD, the ODDD, and the C-terminal nuclear localization signal (NLS) of the native HIF-1α protein. Expression of an EGFP-fusion construct confirmed that deletion of the ODDD results in a protein that is expressed equivalently in air and anoxia. Loss of the C-terminal NLS did not impair nuclear expression of the protein, suggesting the N-terminal NLS alone is sufficient to ensure correct cellular localization to the truncated protein. The fact that the dominant-negative HIF-1α is expressed independently of

TABLE 3

Cell cycle distribution of Hepa-1 wt, Hepa-1 c4, HT1080, and HCT116 cells after 16 h of culture under aerobic or anoxic conditions

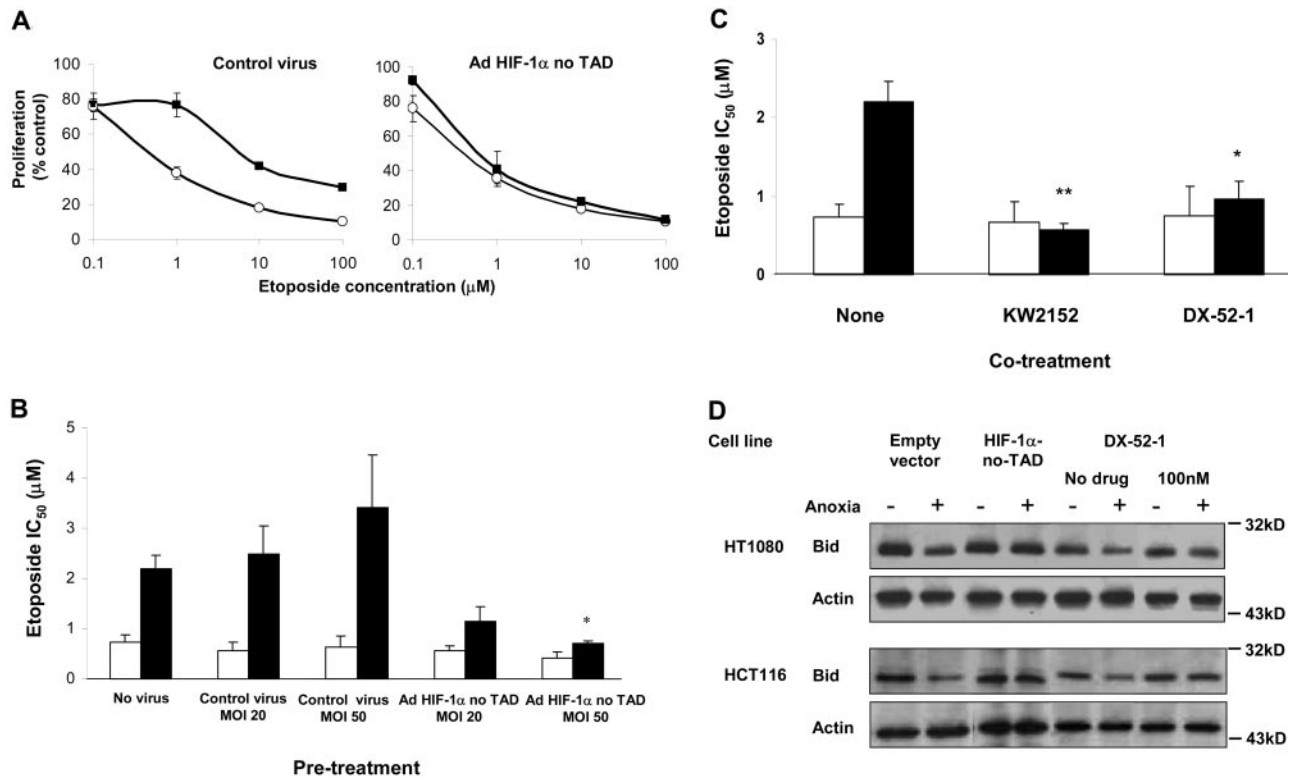| Cell Line	Air			Anoxia		
	G_1	S	G_2M	G_1	S	G_2M
	% population					
Hepa-1 wt	47 ± 12	19 ± 5	22 ± 4	50 ± 15	18 ± 7	17 ± 3
Hepa-1 c4	42 ± 9	18 ± 4	23 ± 4	47 ± 11	18 ± 3	19 ± 4
HT1080	29 ± 3	27 ± 9	45 ± 8	26 ± 4	26 ± 6	48 ± 5
HCT116	39 ± 5	29 ± 5	30 ± 6	32 ± 5	30 ± 7	36 ± 9

Fig. 3. Pretreatment with AdHIF-1α-no-TAD or nontoxic concentrations (100 nM) of KW2152 and DX-52-1 reverses the resistance of HT1080 cells to etoposide treatment under anoxic conditions. Cells were either infected with AdHIF-1α-no-TAD or control virus (Adβ-gal) for 48 h or treated with KW2152 and DX-52-1 for 1 h before and throughout a 16-h exposure to etoposide at a range of concentrations in aerobic (open symbols/bars) or anoxic conditions (closed symbols/bars). Proliferation relative to controls was determined three days later by MTT assay. A, representative survival curves after adenoviral infection at an m.o.i. of 50 from which IC$_{50}$ values were derived. Data presented in A, B, and C are mean values $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$, versus the no virus (B) or control (C) anoxic IC$_{50}$ (two-tailed t test). D, HIF-1α-no-TAD and DX-52-1 pretreatment reverses the HIF-1-dependent down-regulation of Bid expression under anoxic conditions in HT1080 and HCT116 cells. Whole-cell lysates were prepared from transfected/drug-treated cells after 16-h exposure to aerobic ($-$) or anoxic ($+$) conditions. Actin is shown as a loading control. Images are representative of at least two independent experiments.

oxygen tension has important implications in terms of the use of such a protein in a therapeutic context in that this allows the inhibition of HIF-1 regulated by hypoxia and/or as a consequence of oncogenic activation/tumor suppressor inactivation.

In the combination studies, AdHIF-1α-no-TAD pretreatment overcame the hypoxic resistance to etoposide in both HT1080 and HCT116 cells. Evaluation of the small molecule inhibitors supported only the use of KW2152 and DX-52-1 in the combination studies in that the concentrations of topotecan required to inhibit HIF-1-mediated expression were directly cytotoxic in the growth inhibition assay. Furthermore, inhibition of HIF-1 (HRE)-mediated gene expression using topotecan was concomitant with inhibition of constitutive reporter expression suggesting a lack of specificity in the models used. The cytotoxicity profile of KW2152 and DX-52-1 in HT1080 and HCT116 cells was similar, and the IC_{10} was 100 nM or higher. When combined with etoposide, 100 nM KW2152 or DX-52-1 was sufficient to reverse hypoxic etoposide chemoresistance in HT1080 but not in the inherently more resistant HCT116 cells.

We recently identified that the pro-apoptotic protein Bid is down-regulated in a HIF-1-dependent manner under hypoxic conditions (Erler et al., 2004). In the present study, treatment with the dominant-negative HIF-1α ablated this effect in both HT1080 and HCT116 cells. Reversal of the Bid down-regulation could therefore provide the mechanistic basis for chemosensitization observed using the dominant-negative HIF-1α. It is noteworthy that DX-52-1 similarly inhibited the down-regulation of Bid in both cell lines, which correlated with an increase in sub-G_1 cells (data not shown), yet this only modified the hypoxic etoposide response in the HT1080 cells. These data could question the importance of HIF-1-mediated Bid down-regulation in determining chemotherapeutic response or may reflect cell line dependence in the contribution that apoptosis plays in etoposide-mediated cell death. There is also the possibility that they reveal a potential issue with the use of small molecules that inhibit HIF-1 through a mechanism ancillary to their primary target. The absolute specificity of a small molecule is going to dictate the relative importance of their impact on direct HIF-1 targets (e.g., Bid) against nonspecific effects that may be counteractive. Off-target effects mediated by small molecule inhibitors are the focus of ongoing studies. In contrast, adenovirus-mediated delivery of the dominant-negative construct offers direct, specific inhibition that will reduce some of the complexities surrounding drug interactions. Although the use of adenoviral delivery is not without drawbacks, their ability to penetrate through multicellular three-dimensional tumor cell spheroids in vitro (Chadderton et al., 2005) and to transduce hypoxic regions when administered by intratumoral injection to xenografts in vivo (Cowen et al., 2004) supports the contention that AdHIF-1α-no-TAD provides a useful tool with which to fully evaluate the contribution of HIF-1-dependent drug resistance to chemotherapy in experimental models with subsequent clinical indications.

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