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TITLE PAGE

REVERSING HYPOXIC CELL CHEMORESISTANCE *IN VITRO* USING GENETIC AND SMALL MOLECULE APPROACHES TARGETING HYPOXIA INDUCIBLE FACTOR-1

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RUNNING TITLE PAGE

Running Title: HIF-1 INHIBITION AND HYPOXIC CHEMOSENSITISATION

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Non-standard abbreviations:

bHLH, basic-helix-loop-helix; DAPI, 4',6-diamidino-2-phenylindole; EF-1, elongation factor-1-α; EGFP, enhanced green fluorescent protein; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia responsive element; LDH, lactate dehydrogenase; MEF, mouse embryonic fibroblast; MOI, multiplicity of infection; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenytetrazolium bromide; NLS, nuclear localisation signal; ODDD, oxygen dependent degradation domain; PGK, phosphoglycerate kinase; TAD, transactivation domain.

ABSTRACT

The resistance of hypoxic cells to conventional chemotherapy is well documented. Using both adenoviral-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 tumour cells to etoposide. The genetic therapy exploited a truncated HIF-1α protein that acts as a dominant negative (HIF-1α-no-TAD). Its functionality was validated in six human tumour cell lines using HIF-1 reporter assays. An EGFP-fused protein demonstrated that the dominant negative was nuclear localised and constitutively expressed irrespective of oxygen tension. The small molecules studied were quinocarmycin monocitrate (KW2152), its analogue 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 non-toxic (0.1μM; $<IC_{10}$) concentrations of KW2152 and DX-52-1 and exposed to etoposide in air or anoxia (<0.01% oxygen). Topotecan only inhibited HIF-1 activity at cytotoxic concentrations and was not used in the combination study. Etoposide IC_{50} values in anoxia were three-fold higher than those in air for HT1080 (2.2±0.3 versus 0.7±0.2μM) and HCT116 (9±4 versus 3±2μM) cells. KW2152 and DX-52-1 significantly reduced the anoxic etoposide IC_{50} in HT1080 cells whilst only KW2152 yielded sensitisation in HCT116 cells. In contrast AdHIF-1α-no-TAD (MOI 50) ablated the anoxic resistance in both cell lines (IC_{50} values: HT1080, 0.7±0.04μM; HCT116, 3±1μM). HIF-1α-no-TAD expression inhibited HIF-1-mediated down-regulation of the pro-apoptotic 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.

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The resistance of hypoxic cells to anti-cancer therapeutic strategies has a profound impact on treatment response. Within solid tumours hypoxic cell chemoresistance was originally attributed to poor drug distributions and to the contention that hypoxic tumour cells are predominantly quiescent. Pre-clinical 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, 1988; Teicher 1994; Graeber 1996).

More recently a contributory role for the transcription factor, hypoxia-inducible factor-1 (HIF-1) has been revealed (Comerford 2002; Unruh 2003; Erler 2004). HIF-1, a heterodimer composed of HIF-1α and HIF-1β subunits, is a pivotal regulator of gene transcription in response to hypoxia (Wiesner 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 HIF-1α and formation of the HIF-1 complex under hypoxic conditions (Wiesner 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 tumour growth (Jiang 1997; Maxwell 1997; Ryan 1998; Griffiths 2002; Williams 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 tumour suppressor loss (Bardos and Ashcroft 2004). Consistently HIF-1 expression has been recorded in the vast

majority of solid human tumours and has shown positive correlations with both advancing tumour grade and poorer response to therapy (Zhong 1999; Birner 2000; Bos 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. reported that transformed mouse embryonic fibroblasts (MEFs) lacking HIF-1α where more chemo responsive to carboplatin and etoposide than wild type MEFs (Unruh 2003). Here we sought to establish whether HIF-1 targeting using a genetic approach or small molecules is a valid strategy to sensitise human tumour 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 2002). We investigated the impact of the dominant negative and small molecules on the chemotherapeutic response of tumour cells *in vitro*. We used etoposide as a model anticancer agent as we have previously shown that oxygen deprivation results in etoposide resistance (Erler 2004).

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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 1996). The TAD was removed from the HIF-1α cDNA (contained in pBSSKII+) using *Not*1/*Afl*II and a linker inserted (5'-TTAAGTGAGCTTTTTCTTAATCTAGAGC-3'). HIF-1α-no-TAD (1.1kb fragment) was then isolated (*Not*1/*Kpn*1) and cloned into the pcDNA3.1/Zeo vector (Invitrogen, UK) generating pHIF-1α-no-TAD. To construct pHIF-1α-no-TAD-EGFP, the sequence encoding EGFP was removed from pIRES2EGFP (BD Biosciences Clontech, UK) and introduced upstream and in frame of HIF-1α-no-TAD in pBSSKII+. HIF-1α-no-TAD-EGFP was isolated by *Not*1/*Kpn*1 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, USA). 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 co-transformed into BJ5183 competent cells (Stratagene, UK) generating pAdHIF-1α-no-TAD. Primary viral inoculums and large-scale preparations were generated in human embryonic kidney 293 cells (Cowen 2004) and purified using the Adeno-X^{TM} Virus Purification kit (BD Biosciences Clontech, UK). In the experimental studies cells were infected with either Adβ-galactosidase or AdHIF-1α-no-TAD at increasing multiplicities of infection (MOI, number of infectious viral particles per cell) 48h prior to 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 SV40 minimal ($SV40_{min}$) promoter sequence upstream of Firefly *luc*$^{+}$ in the pGL3-promoter vector (Promega, UK). The LDH sequence was cloned in the reverse orientation with respect to the

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promoter. The Carbonic Anhydrase -9 reporter has been described previously (Wycoff 2000) and consisted of the sequence -506/+43 in the native gene cloned into pGL3-basic (Promega, UK).

Cell lines. All of the human tumour cell lines which included HT1080 (fibrosarcoma), DU145 (prostate carcinoma), U87 (glioma), T47D (breast carcinoma), HCT116 and HT29 (colon carcinoma) and the rodent lines were cultured in RPMI (Gibco BRL, UK) containing 10% foetal calf serum and 2mM glutamine in a 95% air: 5% CO_2 environment. All were free from mycoplasma contamination (Mycotect®; Gibco BRL, UK). The CHO-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 1998). The HCT116 HRE-reporter strain expresses the LDH-A $SV40_{min}$ driven Firefly *luc+* cassette detailed above encoded within pCI-neo (Promega, UK). Exposure to anoxia or the hypoxic mimetic cobalt chloride (100μM) for 16h yields a robust (~10 fold) induction of luciferase activity in these cells (Williams and Cowen, unpublished observations). The Hepa-1 wt and HIF-1β-deficient derivative Hepa-1 c4 are murine hepatoma cells and have been extensively described (Maxwell 1997; Ryan 1998; Griffiths 2002; Williams 2002).

Transient transfections. Transfections were performed on exponential phase cells using lipofectamine (Invitrogen, UK) and 1μg of DNA per 2 x 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 luciferase reporter vector, in which renilla *luc+* was cloned upstream of the human elongation factor 1α (EF-1) promoter. The following day transfected cells were sub-cultured into replicate plates and subjected to 16h aerobic or anoxic environment (5% CO_2: 5% H_2: 90% N_2 passed over a palladium catalyst to remove residual oxygen; Bactron anaerobic chamber, Sheldon Manufacturing, USA; oxygen

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concentration <0.01%). Luciferase activity was determined using a manufactured kit (Promega, UK). To analyse EGFP fluorescence following transfection with pIRES2EGFP or pHIF-1α-no-TAD-EGFP, cells were cultured on sterile glass cover slips. Nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI) and relative localisation of green (EGFP) and blue (DAPI) fluorescence analysed using an Axioplan microscope (Zeiss, Germany) and associated software.

Western blot analysis. For EGFP protein detection transfected cells were lysed using the Nuclei EZ Prep Nuclei Isolation kit (Sigma, UK) and nuclear and cytoplasmic samples prepared. Aliquots containing 40μg of protein were run on 10% SDS polyacrylamide gels and then transferred to Hybond membranes (Amersham Biosciences, UK). EGFP-containing proteins were detected using mouse anti-EGFP JL-8 antibody (1:1000; BD Biosciences Clontech, UK) followed by anti-mouse horseradish peroxidase conjugated IgG (1:2500; Sigma, UK), with enhanced chemiluminescence (Amersham Biosciences, UK) used to identify relevant bands. For Bid protein detection whole cell lysates were prepared and 20μg of protein resolved using 15% gels. Bid was detected using 1:1 mixture of 1:500 Santa Cruz Biotechnology (USA) and 1:2000 R & D Systems Europe (UK) antibodies raised in goat (Erler 2004). Actin was revealed using AC40 antibody (1:2000; Sigma, UK).

Carbonic Anhydrase Activity Assay. Carbonic Anhydrase activity was determined as a surrogate marker for HIF-1 function (Wycoff 2000) in whole cell lysates prepared in hypotonic buffer supplemented with protease inhibitors (10mM HEPES, 10mM NaCl, 1mM KH_2PO_4, 5mM $NaHCO_3$, 1mM $CaCl_2$, 0.5mM $MgCl_2$, 1mM PMSF, 10μg ml^{-1} aprotinin, 10μg ml^{-1} leupeptin, 1μg ml^{-1} pepstatin) using the Wilbur-Anderson method (Wilbur and Anderson 1948). The time required (seconds) for a saturated CO_2 solution to lower the pH of 0.02M Tris-HCl from 8.3 to 6.3 was determined as a marker for Carbonic Anhydrase activity. Assays were performed on ice using 0.01mg ml^{-1} lyophilised Carbonic Anhydrase

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(Worthington Biochemical's, UK) as a positive control. Time without enzyme (T_0) was determined by placing 6ml of 0.02M Tris-HCl on ice and recording the pH. 4ml of ice-cold CO_2-saturated water was added and the time taken for the 2-unit pH change recorded. Time with enzyme (T) was ascertained by adding 100µl of the positive control or sample. Carbonic Anhydrase activity (units mg^{-1}) was determined as $2x (T_0 – T) / T$ x mg protein.

Cytotoxicity studies. Cells (untreated or virally infected) were sub-cultured 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, UK), KW2152 (2a,3,4,5,6,6a,7,11b-octahydro-11-methoxy-12-methyl-3,6-imino-1H-2-oxa-11 c- azanaphth(1,2,3-cd)azulene-5-carboxylic acid monocitrate; quinocarmycin citrate), DX-52-1 (NSC-607097; quinocarmycin analogue; National Cancer Institute, USA [Rapisarda 2002]) or topotecan ($C_{23}H_{23}N_3O_5$.ClH; SmithKline-Beecham/Merck Pharmaceuticals, UK) were added at a range of concentrations for 16h. Drug containing medium was replaced with fresh medium and cells cultured for 3 days in standard (aerobic) conditions. The influence of drug exposure on cell proliferation was ascertained by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenytetrazolium bromide) assay as previously described (Cowen 2004). In the combination studies KW2152 and DX-52-1 were added 1h prior to 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 6-well plates at a concentration of 1.5×10^6 per well. The following day the cells were exposed to anoxia or cultured under standard aerobic conditions for 16h. Cells were fixed, RNase-A treated, stained with propidium iodide and analysed by flow cytometry (Becton Dickinson FACSort) using standard techniques (Williams 1996).

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RESULTS

Subcellular localisation and oxygen dependence of HIF-1α-no-TAD. To generate the HIF-1 dominant negative HIF-1α-no-TAD the transactivation domains (TAD) were removed from the coding sequence of human HIF-1α. This deleted the oxygen dependent degradation domain (ODDD) whilst leaving those regions required for dimerisation and DNA binding intact (Fig. 1A). To ease investigation of the sub-cellular localisation of the dominant negative, the coding sequence for enhanced green fluorescent protein (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 hours of air or anoxia. HIF-1α-no-TAD-EGFP localised 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 85kDa 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 (hypoxia-responsive element; HRE) from the lactate dehydrogenase (LDH) promoter. A range of tumour cell lines were transiently transfected with the LDH-HRE reporter construct alone or in combination with pHIF-1α-no-TAD. The dominant negative 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

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expression driven by the HREs from the PGK (phosphoglycerate kinase) 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 SV40 promoter context, HIF-1α no TAD had no significant effect on gene expression regulated by the constitutive human elongation factor-1 (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 following 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 following 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 2005). Carbonic Anhydrase 9 activity in HT29 cells treated with control virus (50 viral particles per cell; MOI 50) was 1.05 ± 0.07 units' mg^{-1} in air and 2.64 ± 0.50 units mg^{-1} following anoxic exposure. AdHIF-1α-no-TAD treatment (MOI 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^{-1} respectively).

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Targeting HIF-1 transactivation using small molecules. Consistent with previous observations using stably transfected U251 human glioma cells (Rapisarda 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 (300nM) was below the concentration causing a 50% reduction in proliferation (IC_{50}) in HCT116 wild-type cells (1.2±0.4μM and 0.7±0.3μM 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 (600nM) than the IC_{50} (0.05±0.04μM). Furthermore the concentration of topotecan required to inhibit constitutive, EF-1 driven reporter expression by 50% was lower than that required for HRE inhibition (100nM). This was not the case for DX-52-1 and KW2152 with 50% inhibition of EF-1-mediated expression achieved using concentrations of 900nM and 800nM respectively.

AdHIF-1α-no-TAD and non-toxic doses of DX-52-1 and KW2152 can reverse the anoxic resistance of tumour 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 two-fold increase in etoposide concentration was required to give the same level of cell kill in anoxic conditions compared 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 as this was not significantly changed compared to 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 16h anoxic exposure used (Table 3). The IC_{50} values following a 16h

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exposure to etoposide under aerobic or anoxic conditions were 0.7 ± 0.2 and 2.2 ± 0.3μM for HT1080 and 3 ± 2 and 9 ± 4μM for HCT116 cells. Pre-treatment with AdHIF-1α-no-TAD decreased the anoxic etoposide IC_{50} concentration. An MOI of 50 was sufficient to sensitise both HT1080 and HCT116 cells to achieve IC_{50} concentrations that mimicked those in air (Fig. 3A and B; Table 2 and data not shown). Relative resistance values (anoxic IC_{50}/aerobic IC_{50}) were calculated from three independent experiments and show that at MOI 50 there was some aerobic etoposide sensitisation in the HT1080 but not HCT116 cells (Table 2). Pre-treatment with control virus had no effect (Fig. 3; Table 2). A non toxic 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 100nM. In addition to causing minimal growth effects, this concentration had no effect on constitutive, EF-1 driven gene expression in either aerobic or anoxic conditions (data not shown). DX-52-1 and KW2152 were dosed at 100nM in combination with etoposide treatment. The direct cytotoxicity of topotecan precluded its use in the combination studies. In HT1080 cells, co-treatment with either drug significantly reduced the IC_{50} for etoposide when exposed under anoxic conditions whilst having no significant effect on the aerobic IC_{50} value (Fig. 3C). KW2152 co-treatment afforded some reduction in the relative resistance of HCT116 cells to etoposide treatment under anoxic conditions, whilst DX-52-1 had no effect (Table 2).

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

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DISCUSSION

HIF-1 has emerged as a target for the development of anti-cancer therapeutics. This was initially underpinned by xenograft studies that established a pro-tumour role for HIF-1 (Maxwell 1997; Ryan 1998; Griffiths 2002; Williams 2002) and supported targeted disruption of HIF-1 transactivation as a strategy to reduce tumour growth (Kung 2000; Chen 2003; Stoeltzing 2004). Compelling supportive data has arisen through the observations that HIF-1 has an influential role in modulating chemotherapeutic response (Unruh 2003; Erler 2004). If HIF-1 targeted approaches could be rationally applied in the context of standard chemotherapy there is potential for a greater therapeutic efficacy than could be achieved through HIF-1 targeting alone. This is particularly in light of the results of studies using admixed populations of HIF-1 wild type and deficient cells demonstrating that 1:100 wild type cells is sufficient to rescue the *in vivo* growth of a HIF-1 deficient population (Hopfl 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 analogue topotecan and DX-52-1, the hydrocyanation product of KW2152) that were revealed as potential HIF-1 inhibitors in the study of Rapisadra (2002). This pilot study in which the NCI diversity set of 2000 compounds was evaluated reported four hits (topotecan, two further camptothecin analogues and DX-52-1) that could inhibit both HRE-reporter and endogenous HIF-1 dependent (vascular endothelial growth factor) expression. Subsequently a number of additional small molecule inhibitors of HIF-1 function have been identified (Yeo 2003; Mabjeesh 2003; Welsh 2004; Tan 2005).

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The generation of the dominant negative involved deleting the N- and C-TAD, the ODDD and the C-terminal nuclear localisation 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 localisation to the truncated protein. The fact that the dominant negative is expressed independently of oxygen tension has important implications in terms of the use of such a protein in a therapeutic context as this allows the inhibition of HIF-1 regulated both by hypoxia and/or as a consequence of oncogenic activation/tumour suppressor inactivation.

In the combination studies AdHIF-1α-no-TAD pre-treatment 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 as 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 100nM or higher. When combined with etoposide, 100nM KW2152 or DX-52-1 was sufficient to reverse hypoxic etoposide chemoresistance in HT1080 but not in the inherently more resistant HCT116 cells.

Recently we have identified that the pro-apoptotic protein Bid is down-regulated in a HIF-1 dependent manner under hypoxic conditions (Erler 2004). In the present study treatment with the dominant negative ablated this effect in both HT1080 and HCT116 cells. Reversal of the Bid down-regulation could therefore provide the mechanistic basis for chemosensitisation observed using the dominant negative. Interestingly, DX-52-1 similarly inhibited the down-

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regulation of Bid in both cell lines, which correlated with an increase in sub G1 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 non-specific effects that may be counteractive. Off target effects mediated by small molecule inhibitors are the focus of ongoing studies. In contrast adenoviral-mediated delivery of the dominant negative construct offers direct, specific inhibition that will reduce some of the complexities surrounding drug interactions. While the use of adenoviral delivery is not without drawbacks, their ability to penetrate through multi-cellular 3-dimensional tumour cell spheroids *in vitro* (Chadderton 2005) and to transduce hypoxic regions when administered by intra-tumoural injection to xenografts *in vivo* (Cowen 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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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. (A) Schematic of HIF-1α and the truncated dominant negative, HIF-1α-no-TAD. (B). HIF-1α-no-TAD is nuclear localised and expressed independently of oxygen availability. C4.5 cells were transfected with pIRESEGFP or pHIF-1α-no-TAD-EGFP and EGFP visualised using fluorescence microscopy. EGFP co-localises with nuclear DAPI staining in pHIF-1α-no-TAD-EGFP expressing cells whereas EGFP encoded by pIRESEGFP is cytoplasmic. (C). Nuclear localisation 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 tumour cells were co-transfected with LDH-HRE-luciferase and either pHIF-1α-no-TAD (closed bars) or blank DNA (open bars), exposed to aerobic or anoxic conditions for 16h and luciferase activity determined. Data shown are average values ±SE [n=3].

Figure 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 (MOI) 48h prior to exposure of cells to air or anoxia for 16h. The data are averages of reporter induction ± SE [n=3]. (B) Topotecan (TPT) and DX-52-1 inhibit cobalt chloride (100μM) mediated induction of luciferase in HCT116 HRE-reporter cells. Drugs were added 1h prior to and maintained throughout the 16h exposure to the hypoxic mimetic cobalt chloride. Data presented were determined from 3 experiments (±SE).

Figure 3. Pre-treatment with AdHIF-1α-no-TAD or non-toxic concentrations (100nM) 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 48h or treated with KW2152 and DX-52-1 for 1h prior to and throughout the etoposide exposure for 16h 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 following adenoviral infection at an MOI of 50 from which IC_{50} values were derived. Data presented in (A, B and C) are mean values ± SE. *$p<0.05$, **$p<0.01$, versus the no virus (B) or control (C) anoxic IC_{50} (two tailed t-test). (D). HIF-1α-no-TAD and DX-52-1 pre-treatment 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 16h exposure to aerobic (-) or anoxic (+) conditions. Actin is shown as a loading control. Images are representative of at least two independent experiments.

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

Cell line	Tumour type	Reporter inhibition (fold[a])							
		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						

[a] 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.

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

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

[b] To gain resistance factor values for HT1080 and HCT116 cells, Ic50 values obtained under each experimental condition were related to the aerobic Ic50 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 ± SEM.

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

Table 3. Cell cycle distribution of Hepa-1 wt, Hepa-1 c4, HT1080 and HCT116 cells following 16h culture in aerobic or anoxic conditions.

Cell line	Cell cycle distribution (% population)					
	Air			Anoxia		
	G_1	S	G_2M	G_1	S	G_2M
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. 1.

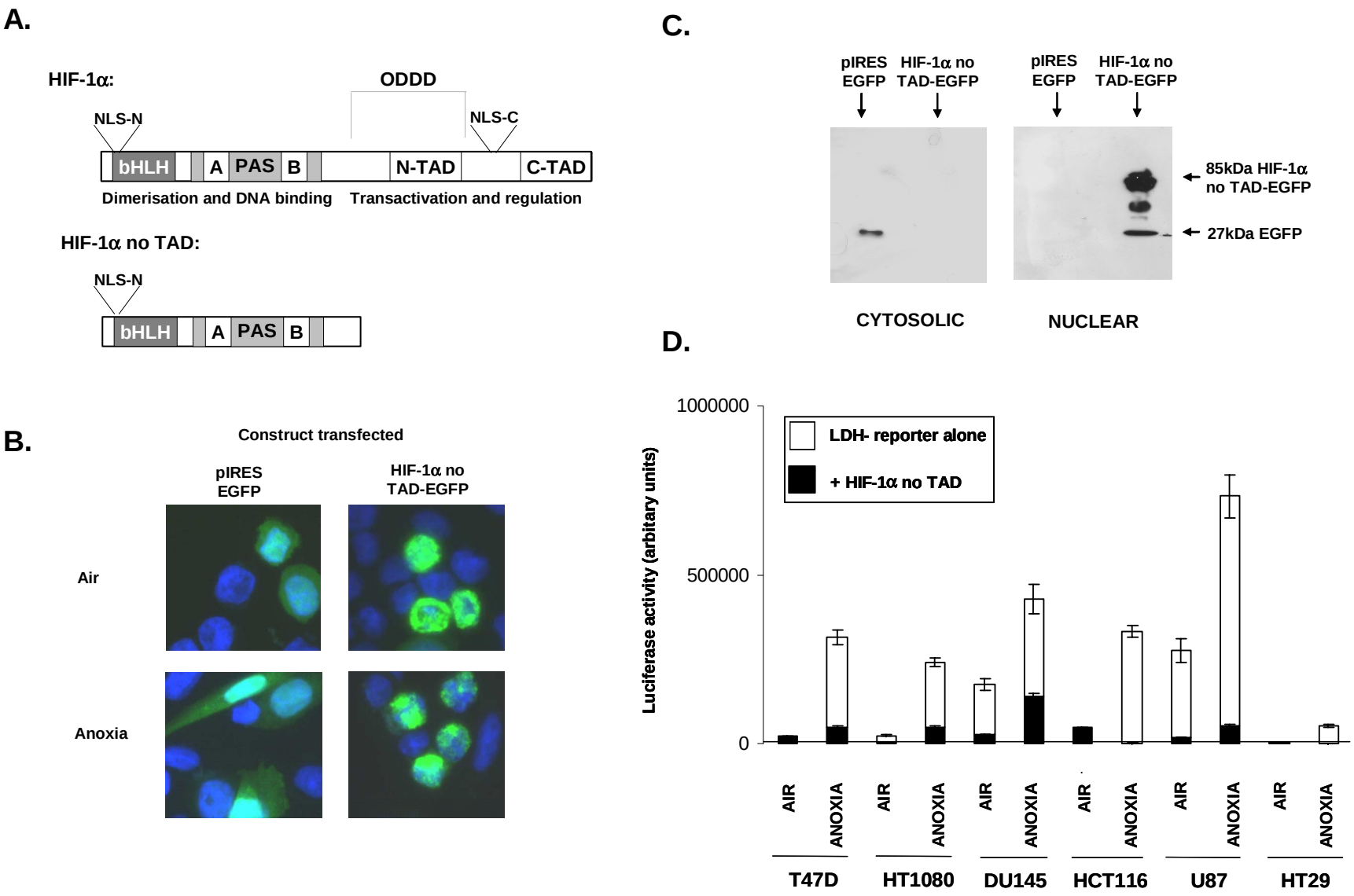

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Fig. 2.

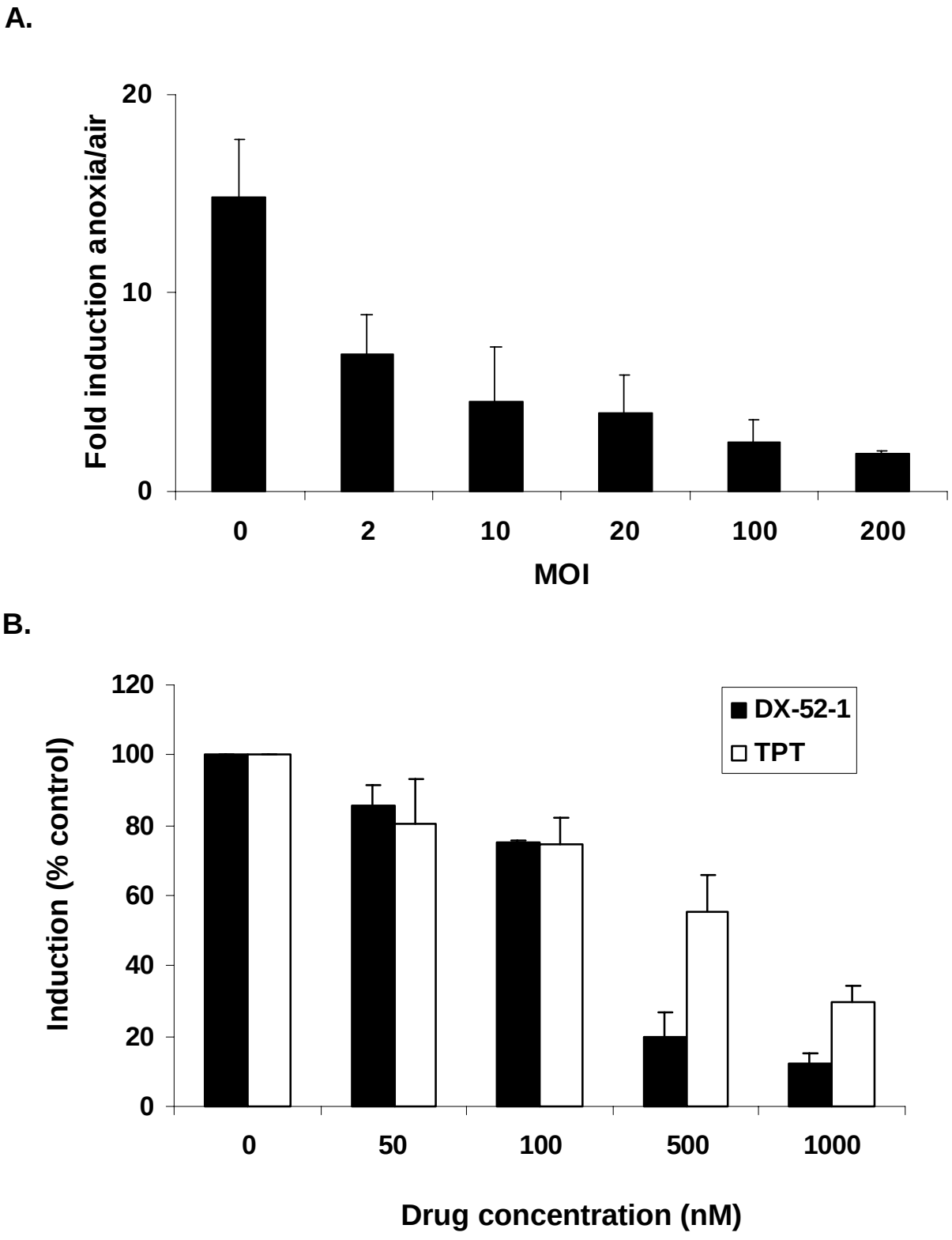

Fig. 3.