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Validation of a Hypoxia-Inducible Factor-1 α Specimen Collection Procedure and Quantitative ELISA in Solid Tumor Tissues

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Abstract

Hypoxia-inducible factor-1 α (HIF-1 α) is an important marker of hypoxia in human tumors and has been implicated in tumor progression. Drugs targeting HIF-1 α are being developed, but the ability to measure drug-induced changes in HIF-1 α is limited by the lability of the protein in normoxia. Our goal was to devise methods for specimen collection and processing that preserve HIF-1 α in solid tumor tissues and to develop and validate a two-site chemiluminescent quantitative ELISA for HIF-1 α . We tested various strategies for HIF-1 α stabilization in solid tumors including nitrogen gas-purged lysis buffer, addition of proteasome inhibitors, or the prolyl hydroxylase inhibitor 2-hydroxyglutarate, and bead homogenization. Degassing and addition of 2-hydroxyglutarate to the collection buffer significantly increased HIF-1 α recovery, while bead-homogenization in sealed tubes improved HIF-1 α recovery and reduced sample variability. Validation of the ELISA demonstrated intra- and inter-assay variability of less than 15% and accuracy of $99.8\% \pm 8.3\%$ as assessed by spike recovery. Inter-laboratory reproducibility was also demonstrated ($R^2 = 0.999$). Careful sample handling techniques allow us to quantitatively detect HIF-1 α in samples as small as 2.5 μ g of total protein extract, and this method is currently being applied to analyze tumor biopsy specimens in early-phase clinical trials.

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Keywords

Hypoxia-inducible factor; HIF-1 alpha; quantitative ELISA; pharmacodynamics; solid tumors

Introduction

Tumor hypoxia is a common feature of a wide spectrum of solid tumors and has long been recognized to drive tumor progression and treatment resistance [1]. One of the most important regulators of the hypoxia response is hypoxia-inducible factor-1 (HIF-1) which mediates transcription of numerous genes involved in biological processes such as angiogenesis, invasion, metastasis, and tumor metabolism [2, 3]. HIF-1 α is overexpressed in many human cancers including malignancies of the brain, breast, colon, lung, ovary, pancreas, prostate, and kidney, and is associated with resistance to treatment and poor prognosis [4–7]. As a result, HIF-1 α is an attractive therapeutic target for cancer therapy, and several drugs that inhibit HIF-1 α are undergoing clinical evaluation. Validation of HIF-1 inhibitors in relevant in vivo models is essential to move these potential therapeutic agents to the clinic, but this effort has been hindered by the absence of established methods to reliably and reproducibly quantify changes in HIF-1 α protein in tumor tissues. Along with the intra-tumoral heterogeneity observed during hypoxia [8], HIF-1 α protein instability in the presence of oxygen limits reliable measurement in samples that are acquired and processed under normoxia [9, 10]. To date, HIF-1 α protein expression has been most commonly assessed by immunohistochemistry (IHC) or Western blot, but the semi-quantitative nature of these techniques or the requirement for relatively large amounts of protein limits their usefulness as sensitive and qualified biomarker assay methods, especially in human biopsy specimens. Alternatively, HIF-1 α activity has been assessed indirectly through protein or mRNA expression levels of HIF-1 target genes such as vascular endothelial growth factor (VEGF) and carbonic anhydrase IX, or surrogate markers such as angiogenesis and micro-vessel density.

Here we report a rigorous process for optimizing specimen collection and processing, and the development and analytical validation of an ELISA for HIF-1 α that addressed sample extraction methods, assay reproducibility across different laboratories, fitness-for-purpose testing in relevant preclinical models, and clinical validation in human specimens [11, 12]. Our method preserves and stabilizes HIF-1 α in solid tumor tissues allowing quantitation of HIF-1 α . The assay has been used to measure drug effect on HIF-1 α protein levels in human xenograft models. Finally, data from human biopsy specimens are presented.

Materials and Methods

Cell lines

Human cell lines PC-3 (prostate adenocarcinoma), DU145 (prostate adenocarcinoma), SiHa (cervical squamous cell carcinoma), A375 (melanoma), and HCT-116 (colon adenocarcinoma) were purchased from and authenticated by the American Type Culture Collection (ATCC) and cultured in appropriate media supplemented with 10% fetal bovine serum (Lonza) and 50 mg/L gentamicin sulfate (Lonza). The identity of each cell line was

confirmed using Identifiler STR genotyping (Applied Biosystems). Cells were cultured for less than 6 months before renewal from early passage, frozen stocks. Cells were maintained at 37°C in a humidified incubator containing 21% O₂ and 5% CO₂ in air (referred to as normoxic conditions). For hypoxic culture, PC-3 cells were placed in a CO₂ incubator flushed with a mixture of gas containing 1% O₂, 5% CO₂, and 94% N₂ for 24 hours (details in Supplemental Methods). Cells were grown to super-confluence in T75 (75 cm²) flasks, washed in cold phosphate-buffered saline (PBS), and immediately lysed in the flask on ice. To increase baseline HIF-1α levels prior to lysis, SiHa cells were treated with 1 μM bortezomib (Fisher Scientific) for 4 hours.

Animal models and drug administration

Female athymic nu/nu (NCr) mice (Frederick National Laboratory for Cancer Research Animal Production Program, Frederick, MD) were implanted with A375, PC-3, DU145, or HCT-116 cells as previously reported [13]. Mice developing tumors served as donors; tumors were maintained by serial in vivo passage using tumor fragment transplantation when the donor tumors reached 10 to 15 mm in diameter. Tumors were staged to a preselected size (weight = 150–300 mg) calculated using the following formula: weight (mg) = (tumor length × tumor width²) / 2 [14]. Mice were housed in sterile, filter-capped, polycarbonate cages (Allentown Caging) maintained in a barrier facility on a 12-hour light/dark cycle and were provided sterilized food and water ad libitum. Mice were randomized into groups before initiation of treatment using a commercial software program (Study Director, Studylog Systems, Inc.).

Topotecan (NSC 609699), the indenoisoquinoline NSC 743400, and selumetinib (AZD6244) were obtained from the Developmental Therapeutics Program, National Cancer Institute (NCI). Topotecan was administered intraperitoneally (IP) in a sterile water vehicle. NSC 743400 was administered intravenously (IV) in a vehicle composed of 10 mM citric acid:5% dextrose (1:3). Selumetinib was administered orally by gavage in a vehicle composed of 10% DMSO. Dose volume was defined as 0.1 mL/10 g body weight. All drugs were administered as a single dose in 0.1 mL vehicle/10 g body weight.

The Frederick National Laboratory for Cancer Research is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and follows the USPHS Policy for the Care and Use of Laboratory Animals. All the studies were conducted according to an approved animal care and use committee protocol in accordance with the procedures outlined in the “Guide for Care and Use of Laboratory Animals” (National Research Council; 1996; National Academy Press; Washington, D.C.).

Xenograft collection

Mice were anesthetized by isoflurane gas inhalation before biopsy or tumor resection. When surgical anesthesia was reached (no toe pinch), the skin was disinfected with Nolvasan (Fort Dodge Laboratories) and a 2 to 5 mm incision was made through the skin adjacent to the subcutaneous tumor being biopsied. An approved 18-gauge human biopsy needle (Temno, Allegiance Healthcare) was passed through the skin incision into the tumor. The wound was closed with a surgical wound clip after sample collection. Collected material (~1 × 5 mm)

was immediately flash frozen either in an empty, sterile o-ring sealed, screw-capped cryovial that was precooled on ice or a tube that was prefilled with degassed lysis buffer. Frozen specimens were stored at -80°C until use. Xenograft tumor quadrants were collected by standard dissection methods, cut into four equal pieces with fine-point scissors and placed into either empty cryovials or tubes prefilled with degassed lysis buffer, pre-cooled on ice as described above. Shallow needle biopsy collections refer to needle passes near the surface of the tumor while deep collections refer to needle collections that passed through the core of the tumor.

Extract preparation

Lysis of all samples used HIF-1 Alpha Cell Extraction Buffer (H-CEB): 50 mM Tris (pH 7.4), 300 mM NaCl, 10% (w/v) glycerol, 3 mM EDTA, 1 mM MgCl_2 , 20 mM (β -glycerophosphate, 25 mM NaF, and 1% Triton X-100. Just before lysis, H-CEB buffer was supplemented with complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich, St. Louis, MO). Degassed buffer was prepared for specimen collection by bubbling nitrogen gas through the buffer at the bottom of the tube using a pipette tip for 3 minutes. The pipette was withdrawn slowly from the tube while filling the head of the tube with nitrogen. The tube was immediately sealed to minimize oxygen seepage back into the tube and then flash-frozen in liquid nitrogen. Validation studies of of pre- and post-degassed buffer dissolved oxygen content can be found in the Supplemental Methods.

Specimens that were collected dry flash-frozen in empty tubes were processed by adding degassed H-CEB directly to the frozen tissue; samples that were collected in degassed H-CEB containing tubes followed by flash-freezing were thawed on ice. Needle biopsy samples were collected in 0.25 mL degassed H-CEB while tumor quadrants and cell pellets were collected into 0.15–0.4 mL depending on sample size. Three different sample homogenization techniques were tested. For sonication, tissue was minced with fine-point scissors then disrupted by sonication at an output of 02–03 watts for 15 to 30 seconds three times (Sonic Dismembrator 550; Fisher Scientific) while the specimen tube was kept on ice. For grinding, cell samples were processed with a Bio-Gen PRO200 homogenizer (PRO Scientific Inc., Oxford, CT) with 7 mm probe for 15 to 30 seconds while the specimen tube was kept on ice. Samples homogenized using bead-beating (Precellys24; Bertin Technologies) were collected in or placed into homogenization tubes prefilled with beads and degassed H-CEB, and homogenized at $6,000\text{ rpm} \times 15\text{ s} \times 2\text{ cycles}$. Following homogenization, extracts were supplemented with sodium dodecyl sulfate (SDS) (Sigma-Aldrich) to a final concentration of 1%, vortexed, and then either kept on ice for 20–30 minutes, or immersed in a 56°C water bath for 20 minutes or a boiling water bath for 5 minutes depending on the experiment. Subsequently, specimens were clarified by centrifugation at $13,000 \times g$ for 10–15 minutes at 2°C to 8°C .

ELISA control lysate preparation

Assay control lysates were produced from $1\text{ }\mu\text{g}/\mu\text{L}$ extracts of HEK293T cells transfected with a HIF-1 α TrueORF cDNA clone (OriGene Technology, Inc.) and PC-3 cells. Extracts were mixed in different ratios to obtain readings at critical points in the standard curve; three

control lysate levels (high, mid, and low) of approximately 725, 240, and 50 pg/mL HIF-1 α , respectively, were used. Each control lysate was loaded at 10 μ g/well. New lots of assay control lysates were subject to analysis using a proficiency testing panel that included the previous passing lot of control lysates; acceptance criteria was $\pm$ 25% variance from the previous passing lot.

ELISA procedure

The HIF-1 α ELISA used the R&D Systems human/mouse total HIF-1 α DuoSet IC kit for development of 96-well plate ELISA assays with the following modifications. The kit-supplied HIF-1 α capture monoclonal antibody was reconstituted to 500 μ g/mL in 1X PBS and the HIF-1 α detection polyclonal antibody was reconstituted to 5 μ g/mL in 1X PBS containing 0.05% Tween-20 and 5% BSA (referred to hereafter as reagent diluent). The recombinant human HIF-1 α peptide included in the kit was reconstituted to 100 ng/mL in reagent diluent, and then serially diluted 1:2 from 1000 to 7.8 pg/mL in reagent diluent for the standard curve. Protein concentration was determined for all unknown sample tissue extract using a Bicinchoninic Acid (BCA) Assay Kit (Thermo Scientific Pierce; Rockford, IL) adapted for use in a 96-well plate according to the manufacturer's instructions. Extracts were diluted in reagent diluent to a final protein concentration of 0.5 or 1 μ g/ μ L for the assay, and further diluted if necessary.

Reacti-Bind white opaque 96-well plates (Thermo Scientific Pierce) were coated with 4 μ g/mL mouse monoclonal anti- HIF-1 α antibody diluted in 1X PBS at 100 μ L per well (Lot KQE0411012), covered with acetate sealers (Thermo LabSystems; Franklin, MA), and incubated at 2°C to 8°C overnight (16 ± 1 hour). The plates were then washed with 400 μ L/well 1XPBS with 0.05% (w/v) Tween 20 (referred to hereafter as wash buffer) three times on a Bio-Tek ELx405 Select plate washer (Bio-Tek Instruments, Inc.), and blocked with 300 μ L/well reagent diluent for 1–2 hours at 25°C $\pm$ 3°C.

Plates were then washed three times as described above, and 100 μ L of each standard (in duplicate), 10 μ L of diluted samples plus 90 μ L of reagent diluent per well (in duplicate or triplicate), and 10 μ L of control cell extracts plus 90 μ L of reagent diluent per well (in duplicate or quadruplicate), were loaded onto the plate for a final assay well volume of 100 μ L. Blank wells were filled with reagent diluent only. An optimal map analyzing clinical samples in a 96-well plate format places the control extracts in duplicate on either side of the plate (4 wells each in total), standard wells down the middle of the plate in duplicate, and unknown samples in triplicate between the controls and standards. Blank wells are placed in the top and bottom row of the plate; corner wells are not used when determining background levels for the assay.

The plates were covered with fresh acetate sheets and incubated at 2°C to 8°C overnight. Biotinylated goat polyclonal anti-HIF-1 α antibody was prepared in reagent diluent at 100 ng/mL (Lot KKR0311012). Plates were again washed three times with wash buffer and 100 μ L polyclonal antibody was added per well. The plates were incubated at 25°C $\pm$ 3°C for 2 hours and washed three times with wash buffer. Then, 100 μ L streptavidin conjugated horseradish peroxidase (R & D Systems) diluted at 1:200 (Lot 1260918) in reagent diluent was added to each well and incubated in the dark for 30 minutes at 25°C $\pm$ 3°C. At the end

of the conjugate incubation, plates were washed three times in wash buffer and 100 μ L LumiGLO Chemiluminescent Substrate (KPL) was added to each well. Plates were immediately read on a Tecan Infinite M200 plate reader (Tecan; Mannedorf, Switzerland). Relative light unit values were plotted using a HIF-1 α analysis template to generate standard curves. Average HIF-1 α level (minus background), standard deviation (SD), and coefficient of variation (CV) for each assay control and unknown sample was determined using the HIF-1 α standard curve. Final HIF-1 α readout was reported as pg HIF-1 α per 1 μ g total protein using the HIF-1 α standard curve.

Antibody specificity of the kit-supplied reagents was tested by Western blot analysis in PC-3 cells incubated in normoxic or hypoxic conditions. Each new lot of HIF-1 α ELISA kits from R&D Systems was subject to a validation protocol for analytical performance.

Assay validation

Quantitative validation was carried out to establish assay accuracy and precision; accuracy was evaluated by spike/recovery of recombinant HIF-1 α standards in tumor biopsy extracts and by analysis of dilution linearity of specimens spiked with recombinant HIF-1 α . Intra- and inter-plate assay precision was established by measuring intra- and inter- plate performance with appropriate controls and HIF-1 α standards (7.8, 15.6, 31.3, 62.5, 125, 250, 500, and 1000 pg/mL) on a total of six plates run on three separate days. Inter-operator, inter-site, and inter-day precision was measured across two independent NCI laboratories, the National Clinical Target Validation Laboratory (NCTVL) and the Pharmacodynamic Assay Development and Implementation Section (PADIS). A total of 18 matched extract samples including PC-3 xenografts from untreated mice, SiHa cells treated with 1 μ M bortezomib for 2 and 3 hours, and the three control lysates were divided between the two laboratories for processing and the entire set was analyzed by each laboratory with the HIF-1 α ELISA. Assay specificity was checked by Western blotting of cell extracts to confirm specific reactivity with HIF-1 α .

Recovery experiments were performed as follows: recombinant HIF-1 α was prepared in reagent diluent as for a standard curve determination and was then spiked into extracts from two HCT-116 xenografts from untreated mice at a final concentration of 15.63, 31.3, 62.5, 125, 250, and 500 pg/mL recombinant HIF-1 α . Extracts were pre-diluted in reagent diluent to 2.5, 5, and 10 μ g protein per 10 μ L. Extracts were added to wells containing either 90 μ L of the assay diluent alone or 40 μ L of the assay diluent combined with 50 μ L of recombinant HIF-1 α standards at the concentrations listed above in duplicate wells, and then assayed as described in the methods section. Assay controls and standards were run on each plate and recovery experiments were performed twice. HCT-116 xenograft extracts, dry flash frozen and lysed in degassed buffer, were used to test for dilution linearity of HIF-1 α recovery by loading 1.25 to 20 μ g total protein per well (as determined by the BCA assay) and assaying with the HIF-1 α ELISA. Sample readouts were normalized to pg HIF-1 α per 1 μ g total protein per well; all 1.25 μ g loads were below the LLQ of the assay. HIF-1 α molecules per cell were calculated from the immunoassay results assuming an assay recovery of 100%, 1×10^6 cells per assay, and a molecular weight of 92.67 kD,

Western blotting

Protein concentrations were determined by BCA assay, and cell lysate loads up to 40 µg per well were run on 4% to 20% precast polyacrylamide gradient gels (Bio-Rad Laboratories) for SDS-PAGE at 100 V for 2 hours. Protein was then transferred to nitrocellulose membranes at 100 V for 2 hours. Membranes were blocked overnight at 2°C to 8°C with blocking buffer (LI-COR, LI-COR Biosciences) then probed first with biotinylated goat anti-HIF-1α polyclonal antibody (R&D Systems) diluted to 1 µg/mL with LI-COR blocking buffer overnight at 2°C to 8°C. The blot was then probed with IRDye 680 streptavidin (diluted to 0.1 µg/mL; LI-COR) for 1 hour at 25°C ± 3°C in the dark. Blots were scanned on the Odyssey IR imager (LI-COR).

2-HG enantiomer studies

These experiments used the same extract preparation outlined above except that they included 2-hydroxyglutarate (2-HG) from the following sources: D-(R)-2-HG (3BSC, Cat# 3B3-053228, lot# WH20100114), D-(R)-2-HG (Sigma-Aldrich, Cat# H8378, lot# SLBB6158V), and L-(S)-2-HG (Sigma-Aldrich, Cat# 90790, lot# BCBG2238V). To increase baseline HIF-1α levels prior to lysis, SiHa cells were treated with 1 µM bortezomib (Fisher Scientific) for 4 hours.

Patient biopsy collection

Needle biopsies (18-gauge) were collected from patients with cancer (various types of solid tumors) at the Center for Cancer Research, NCI (Bethesda, MD). Biopsies were collected into o-ring sealed, screw-capped cryovial, immediately dry flash frozen on liquid nitrogen or a dry ice/ethanol bath per our previously published method [13] and stored at -80°C. At the time of the assay, samples were thawed in 250 µL degassed H-CEB and homogenized using ceramic beads. All patients gave written informed consent for study inclusion and were enrolled on NCI institutional review board-approved protocols. The study was performed in accordance with the precepts established by the Helsinki Declaration. The study design and conduct complied with all applicable regulations, guidances, and local policies and was approved by the NCI institutional review board.

Statistical analysis

Regression analysis, Student's unpaired *t*-test (2-tailed), and descriptive statistics including mean, SD, and CV were conducted using Microsoft Excel and GraphPad Prism software. All tests were two-tailed with the statistical significance level (α) set to $P = 0.05$ (95% confidence level).

Results

ELISA assay development and analytical validation

Development of an analytically-validated HIF-1α enzyme-linked immunosorbent assay (ELISA) began with an initial focus on stabilization of HIF-1α in tissue extracts. Once stabilized extracts could be reproducibly isolated, we adapted the R&D Systems 96-well plate ELISA kit for detection of human/mouse total HIF-1α for use on the extracts. The kit-

provided recombinant HIF-1 α was used to prepare a standard curve with a dynamic range of 7.8–1000 pg/mL and a high degree of correlation ($R^2 = 0.99$; Fig. 1A). The lower limit of quantitation (LLQ) for the assay was set at 7.8 pg/mL, and the limit of detection, defined as the mean plus 2 SD of the background calculated from 8 replicates was 3.0 pg/mL. Optimal antibody dilutions, times, and temperatures were determined for each step of the ELISA using the kit-provided reagents. Accuracy was confirmed by spike recovery of cloned protein into cell extracts, and dilution linearity methods to be $99.8\% \pm 8.3\%$ within the extract protein load range of 2.5 to 10 μ g total protein per assay well, and total assay imprecision was less than 10% (Table 1). High, mid, and low assay control lysates were developed to fall within the linear range of the standard curve; control samples across 18 assay plates averaged 719 ± 37 , 238 ± 12 , and 51 ± 5 pg/mL, respectively (Fig. 1A). Optimal protein loads for the assay were determined by specimen dilution linearity which was observed in the range of 5 μ g to 10 μ g protein per well (Fig. 1B).

Assay precision was established by measuring intra- and inter- plate performance with HIF-1 α standards and controls on a total of six plates run on three separate days (Fig. 1C). Mean intra-assay variability (%CV) for the high, medium, and low controls was 2.7%, 4.3%, and 5.3%, respectively, and ranged from 1.1% to 6.3% for the standards. Mean inter-assay %CV for the high, medium, and low controls was 3.8%, 3.2%, and 10.2%, respectively, and 3.0% to 10.5% for the standards. Inter-laboratory reproducibility of the adapted HIF-1 α ELISA was assessed at two independent NCI laboratories, NCTVL and PADIS, with 18 matched extract samples. The sample set was divided between two laboratories for processing, and then the assay was performed independently by each laboratory using the complete set of samples; regression analysis showed a high degree of correlation between the sites with a mean inter-laboratory %CV of 10.4% ($R^2 = 0.999$; Fig. 1D).

Optimization of sample collection and processing methods

The effect of hypoxia on intracellular expression of HIF-1 α protein during cell growth and cell lysis was determined by Western blotting PC-3 cells. As expected, HIF-1 α protein was undetectable or detectable only at very low levels when PC-3 cells were grown and lysed in normoxic conditions (Fig. 2A and 2B); similarly, cells grown in hypoxia, but lysed in normoxic conditions also had very low HIF-1 α by Western blot. To simulate lysis in hypoxic conditions, cells were collected in a hypoxic chamber and placed in H-CEB buffer; lysis in these conditions stabilized HIF-1 α levels in cells grown in hypoxic conditions (Fig. 2A). To determine if freezing of cells would affect HIF-1 α levels, cells were grown in hypoxic conditions, collected and flash-frozen in a hypoxic chamber and stored for 1 hour or 5 days. These frozen lysates yielded amounts of HIF-1 α similar to that obtained from cells cultured in hypoxia and immediately lysed in degassed buffer (Fig. 2B). These Western blot results suggested that the critical step for HIF-1 α stabilization was specimen collection into hypoxic conditions or immediate lysis in hypoxic conditions; after this point, the specimens are stable and can be handled in a manner consistent with laboratory procedures for oxygen-insensitive proteins. Freezing of samples in normoxic conditions results in loss of analyte.

In contrast to the Western blots, the quantitative ELISA has increased sensitivity and could measure HIF-1 α higher yields protein levels in PC-3 cells grown in both normoxic and

hypoxic culture conditions and immediately lysed under normoxic conditions at protein loads of 1.25 to 10 μ g (Fig. 2C). HIF-1 α levels were 2–4 times higher in cells cultured in hypoxic conditions, and HIF-1 α levels in the lowest protein load (1.25 μ g) under normoxic conditions were below the LLQ of the assay. Two different cell disruption methods were initially tested, and sonication was more effective than grinding with a hand-held homogenizer at preserving HIF-1 α analyte in the collected cells (paired *t*-test, *P* = 0.068). As expected, hypoxic culture of PC-3 cells resulted in elevated HIF-1 α levels compared to normoxic samples even when assay read-outs were normalized to total protein load, actin read out, and cell number (Table 2).

As proof that increased HIF-1 α levels could be detected with the ELISA method, SiHa and PC3 cells were treated with 1 μ M bortezomib for 0 to 24 hours, lysed in degassed H-CEB containing 100 μ M bortezomib, and assayed for changes in HIF-1 α levels (Fig. 2C). Bortezomib has previously been reported to increase HIF-1 α levels [15]; both cell lines had a significant increase in HIF-1 α compared to baseline following 4 hours of bortezomib treatment. To further optimize the ELISA, we evaluated whether different homogenization methods affect HIF-1 α yield; as previously demonstrated, sonication gave higher HIF-1 α yields than grinding (Fig. 2D). The increased recovery levels of HIF-1 α observed for the sonicated specimens are most likely due to a decrease in target degradation as a result of use of a metal tissue grinder. This is consistent with the decreased sample variability observed when comparing homogenization with ceramic beads to stainless steel beads (Fig. 2E). We suspect leaching of metals from the chrome plating caused by the mechanical grinding and elution due to interaction with chemicals in the lysis buffer, which is at slightly alkaline pH 7.4.

Next, we compared sonication versus bead-beating homogenization using matched pieces from HCT-116 xenografts in untreated mice. Whereas sonication required mincing the tissue specimens beforehand, bead homogenization using ceramic or metal beads enabled specimens to be extracted into a sealed tube prefilled with beads and degassed H-CEB, thus reducing oxygen exposure and preventing sample cross contamination. Bead-beating homogenization resulted in significantly higher yields of HIF-1 α than sonication, regardless of the size or type of bead (Fig. 2E; *P* < 0.05). The size and type of bead was important to individual sample variability; 1.4-mm ceramic beads had the lowest variability with a 10.3% CV while variability was higher with 2.8-mm ceramic beads (%CV, 15.3%), 2.8-mm metal beads (%CV, 20.0%), and sonication (%CV, 26.3%). Based on the consistency of data across repeated experiments, use of either the 1.4 or 2.8 mm ceramic beads in conjunction with degassed H-CEB tubes is the preferred method for tissue homogenization.

Because obtaining in vivo specimens under hypoxic conditions and freezing samples in a hypoxic chamber are not feasible in the clinical setting, we evaluated several strategies to stabilize HIF-1 α in tumor samples collected in normoxia using xenograft tumor quadrants collected from live mice. We modeled clinical sample collection with 18-gauge needle biopsies of DU145 xenografts from untreated mice and compared HIF-1 α yield from standard dry flash freezing in an empty sterile, normoxic cryovial against flash freezing after collection into a vial containing degassed H-CEB. Collecting specimens in degassed H-CEB to reduce oxygen exposure before freezing significantly increased HIF-1 α recovery

compared to dry, flash-freezing alone ($P = 0.004$) (Fig. 3A). There was no difference in HIF-1 α levels from deep versus shallow tumor biopsy samples (Fig. 3B).

To further improve the HIF-1 α yield, we tested the addition of chemical inhibitors of the HIF-1 α degradation pathway including the prolyl hydroxylase (PHD) inhibitor 2-HG ([R]-enantiomer) and proteasome inhibitors bortezomib and MG132. To evaluate the role of these inhibitors in preventing HIF-1 α degradation, SiHa cells were first incubated with 1 μ M bortezomib for 4 hours to increase baseline HIF-1 α levels and then harvested with degassed H-CEB alone or containing 100 μ M bortezomib, MG132, or (R)-2-HG. Inclusion of any of the three agents in the H-CEB increased the average recovery of HIF-1 α compared to H-CEB alone, though only addition of (R)-2-HG was statistically significant (unpaired t -test, $P = 0.045$; Fig. 3C). A similar analysis was conducted using tumor pieces from the same A375 xenograft quadrants untreated mice collected in H-CEB containing 2-HG, bortezomib, or the combination; H-CEB with 2-HG significantly increased HIF-1 α recovery from A375 xenograft samples compared to H-CEB alone (paired t -test, $P = 0.009$); combination of bortezomib and 2-HG did not increase HIF-1 α recovery (Fig. 3D). We repeated the SiHa experiment to evaluate activity of the (R)-enantiomer on HIF-1 α levels versus the (S)-enantiomer. Since our original supplier of 2-HG (3B Scientific Corporation; 3BSC) could only supply the (R)-enantiomer, (R)- and (S)-enantiomers were obtained from Sigma-Aldrich; in addition, a second lot of the 3BSC (R)-2-HG was tested. Both the (R)- and (S)-enantiomers increased the mean recovery of HIF-1 α compared to baseline. However, while not significant, the new lot of 3BSC (R)-2HG appeared to decrease the ability to recover HIF-1 α (Fig. 2E). A consistent observation in this work, and the principal value of the 2-HG and buffer degassing, was the decrease in variance of measurements within and across xenograft pieces within treatment groups. Of note, baseline HIF-1 α levels were 10-fold lower in the A375 xenograft samples than that observed in the bortezomib pre-treated SiHa cells; we postulate that the improved HIF-1 α yield with addition of 2-HG may only be obvious in tissues where the baseline HIF-1 α levels are elevated. Inactivation of HIF-1 α -degrading enzymes by heat-treatment of lysates was also tested, but HIF-1 α yield was not improved after either boiling lysates for 5 minutes or heat-treating at 56°C for 20 minutes. In addition, boiling abolished the stabilizing effects of 2-HG and the other proteasome inhibitors (data not shown).

In vivo modeling of pharmacodynamic marker performance

Fitness-for-purpose modeling of the assay to use HIF-1 α as a pharmacodynamic biomarker was determined by measuring HIF-1 α levels in xenograft models treated with vehicle or known HIF-1 α -targeting drugs. We selected the topoisomerase I inhibitors topotecan and indenoisoquinoline (NSC 743400) because these agents are non-selective HIF-1 α inhibitors [16–20]. HIF-1 α was measured in A375 xenografts from mice treated with vehicle (water) daily QDx5; 25 mg/kg NSC 743400 QDx1; 25, 12.5, or 2.5 mg/kg NSC 743400 QDx5; or 4 mg/kg topotecan QDx5 (3 animals per group). Whole xenografts were surgically excised 1 hour after the last dose, and HIF-1 α was measured in one quadrant of the excised specimen. Baseline levels of HIF-1 α in the vehicle control animals ranged from 0.25 to 0.78 pg/ μ g protein. HIF-1 α levels dropped by nearly 80% in the 2.5 mg/kg NSC 743400 treated group

and to below the LLQ of the assay in all other treated groups compared to the vehicle control (unpaired *t*-test, $P < 0.05$; Fig. 4A).

HIF-1 α was also measured in PC-3 xenografts from mice treated with vehicle (water) QDx2 ($n = 4$), vehicle QDx5 ($n = 4$), topotecan 4 mg/kg QDx2 ($n = 8$), topotecan 4 mg/kg QDx5 ($n = 8$), or topotecan 1.5 mg/kg QDx5 ($n = 8$). Whole xenografts were surgically excised 7 hours after the last dose and HIF-1 α measured in one quadrant of the excised specimen. Baseline levels of HIF-1 α in the vehicle control animals ranged from 0.43 to 0.56 pg/ μ g protein (QDx2) and 0.43 to 0.96 pg/ μ g protein (QDx5). HIF-1 α levels were only significantly decreased (34%, $P = 0.03$, unpaired *t*-test) in PC-3 xenografts of mice receiving the 4 mg/kg topotecan for 5 days when compared to vehicle (Fig. 4B). This same treatment in mice bearing A375 xenografts resulted in HIF-1 α levels below the LLQ of the assay. No significant change in HIF-1 α levels compared to vehicle was observed for mice treated for 5 days at the lower dose of topotecan, or when the higher dose of topotecan was utilized for 2 days.

Next, we evaluated the effect of inhibiting the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway, one of the major up-stream signaling mechanisms activating HIF-1 α , on HIF-1 α levels [21, 22]. HIF-1 α was measured in 18-gauge needle biopsies from HCT-116 xenografts treated with a single dose of vehicle (DMSO) or 50 mg/kg selumetinib, a potent and selective inhibitor of MAPK kinase (MEK) 1/2. Levels of HIF-1 α significantly decreased 4 hours following administration of selumetinib compared with the DMSO control ($P = 0.03$, unpaired, *t*-test), reaching a maximum inhibition of 42% by 7 hours post-dose, and then recovering by 24 hours after dosing (Fig. 4C).

Clinical validation in patient tumor biopsy specimens

We established that our validated specimen handling and assay method could measure HIF-1 α in human tumor biopsy samples. Samples were from seven patients with advanced cancer; the tumor tissues had been stored at -80°C for 2.3 to 19.6 months after being dry flash frozen under normoxic conditions; samples were thawed and lysed using ceramic beads in 250 μL H-CEB buffer. Levels of HIF-1 α in all patient samples were above the assay LLQ and demonstrated substantial baseline variability, ranging from 0.1 to 1.28 pg/ μ g protein (Table 3). No association between the storage duration of samples and the HIF-1 α levels was found. The average amount of protein in twenty-seven 18-gauge needle biopsy specimens obtained from patients with various solid tumors enrolled in early phase clinical trials at the NCI was 432.2 μg (range, 80–1047.5 μg ; data not shown).

Discussion

Development of novel agents targeting the hypoxic pathway is a rapidly expanding area of developmental therapeutics, and HIF-1 α is one of the most actively investigated therapeutic targets. Different strategies to inhibit HIF-1 α have been developed, including targeting HIF-1 α mRNA expression, protein translation, protein degradation, DNA binding, or transcriptional activity [23]. Either directly or indirectly, these therapeutic strategies aim to modulate HIF-1 α levels, which must be demonstrated in proof-of-mechanism studies in

relevant preclinical in vivo models and, more importantly, in patients with cancer. However, validation of HIF-1 α inhibitors in preclinical and clinical models has been hindered by a lack of established pharmacodynamic assays necessary to demonstrate inhibition of the intended target, HIF-1 α , in tumor tissue.

We developed and analytically validated a method for specimen collection and handling and a quantitative ELISA to measure HIF-1 α in solid tumor extracts; we have also established fitness-for-purpose using xenografts treated with HIF-1 α inhibitors such as topoisomerase I inhibitors and a MAPK/ERK pathway inhibitor. A major obstacle in measuring HIF-1 α levels for in vivo specimens is the lability of HIF-1 α protein in the presence of oxygen during sample collection. HIF-1 α is among the most rapidly degraded proteins under normoxia; upon re-oxygenation, hypoxia-induced HIF-1 α has a half-life of 1–5 minutes [9, 10]. The ability to measure higher HIF-1 α values at baseline, and in samples in general, is critical for assay utility as it increases the dynamic range for measuring HIF-1 α reduction after treatment with HIF-1 α inhibitors. Therefore, as part of assay development, we needed to establish specimen collection and processing methods to prevent rapid degradation of HIF-1 α under normoxic conditions.

Various strategies for HIF-1 α stabilization were tested targeting the main components of the degradation pathway that is activated in the presence of oxygen [24]: 1) specimen collection in nitrogen gas-purged degassed H-CEB to reduce oxygen exposure in the collection stage before flash freezing; 2) addition of the PHD inhibitor 2-HG in H-CEB to prevent hydroxylation of HIF-1 α by PHDs in the early stage of its degradation pathway; 3) addition of a proteasome inhibitor in H-CEB to prevent proteasomal degradation in the late stage of the degradation pathway; 4) bead-beating homogenization using sealed tubes to reduce the influx of oxygen during homogenization; and 5) heat-treating tumor extracts to inactivate all enzymes involved in HIF-1 α degradation. We found that lysis of cells in degassed buffer and use of bead-beating homogenization significantly increased the yield of HIF-1 α in tumor specimens. Although we did not measure the residual oxygen content in the buffer after degassing, purging with inert gaseous nitrogen has been shown to be both a quick and effective procedure to substitute oxygen dissolved in water by nitrogen [25].

HIF-1 α protein is hydroxylated at two proline residues on positions 402 and 564 by PHDs and is then targeted for proteasomal degradation [26]. PHDs require oxygen and 2-oxoglutarate as substrates and depend upon Fe²⁺ and ascorbate as cofactors [26]. Because of its high degree of structural similarity to 2-oxoglutarate, 2-HG can antagonize the binding of 2-oxoglutarate to PHDs and inhibit the activity of PHDs, consequently stabilizing HIF-1 α protein [27]. Cobalt chloride has been widely used as a mimetic of hypoxia based on studies reporting cobalt stabilization of HIF-1 α by inhibiting PHDs through substitution of iron or depletion of ascorbate leading to the Fe³⁺ oxidation state [28, 29]; however, in our ELISA, the addition of 100 μ M cobalt chloride to H-CEB significantly decreased HIF-1 α yield in PC-3 cells compared to buffer alone, probably because of assay interference (data not shown). During assay development high intra-group variability was observed; in addition to use of degassed buffer and bead-beating homogenization, 2-HG was added to the H-CEB buffer to increase stabilization of HIF-1 α protein during extraction, and to minimize variability and the impact of operator-to-operator sample handling. The use of bead-beating

homogenization, compared to sonication, improved HIF-1 α recovery and reduced sample variability, which is of importance because it enabled the detection of even modest drug-induced changes in our pharmacodynamic end point [12].

Assay validation studies, including spike-recovery, dilution-linearity, and assay precision show that this is a reproducible, reliable, and quantitative method. Although other methods have been developed to evaluate the change in HIF-1 α protein levels, such as Western blot and immunohistochemical analyses, our ELISA assay can quantitatively measure HIF-1 α protein levels even in samples collected under normoxic conditions from as little as 2.5 μ g total protein extract with a wide dynamic range, making this assay clinically useful.

Using human melanoma, prostate, and colon cancer xenograft models, we demonstrated that the assay can quantify the pharmacodynamic effects of HIF-1 α -modulating drug treatments. In A375 xenografts, HIF-1 α levels were decreased after treatment with the topoisomerase I inhibitors topotecan and indenoisoquinoline NSC 743400. This result is consistent with previous reports showing that topotecan and NSC 706744, a non-camptothecin, topoisomerase I inhibitor, decreased HIF-1 α expression in preclinical models as assessed by Western blot or immunohistochemistry [16–19]. Complete inhibition of HIF-1 α was also measured by immunohistochemistry in biopsy specimens from some refractory solid cancer patients after treatment with topotecan [20]. Similarly, topotecan treatment significantly decreased levels of HIF-1 α in PC-3 xenografts, but to a lesser extent than in A375 xenografts. This difference in the extent of topoisomerase I-induced HIF-1 α inhibition in PC-3 cells could be due to lower baseline Top1 levels compared to A375 cells [30]. Another possibility could be the constitutive activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, via loss of PTEN, which has been implicated in HIF-1 α regulation [31–34].

Our ELISA also demonstrated that treatment with the MEK1/2 inhibitor selumetinib in the HCT-116 colon cancer xenograft model decreased HIF-1 α levels. This result confirmed that the ELISA can quantify HIF-1 α in the limited material that can be obtained from 18-gauge needle biopsies; the result is consistent with a previous study reporting decreased HIF-1 α levels, in conjunction with decreased phospho-ERK expression, in selumetinib-treated mice bearing Calu-6 DC colon cancer xenografts [35]. Of note, after an initial slight increase in HIF-1 α levels 2 hours post-dose, levels started to decrease by 4 hours and were maximally inhibited 7 hours post-dose; HIF-1 α levels recovered to baseline by 24 hours post-dose. Understanding the time course for inhibition of HIF-1 α and its recovery after treatment is critical both for determining a treatment schedule and establishing optimal time points for collecting tumor biopsies in the clinic. Thus, we believe that our quantitative ELISA has advantages over immunohistochemistry and Western blot for assessing changes in HIF-1 α over time for a specific treatment in relevant preclinical models mirroring clinical trials.

The validated assay and specimen handling standard operating procedures were successfully applied to clinical tumor specimens, establishing that HIF-1 α could be measured in human biopsy samples stored at – 80°C after being dry flash frozen. These assay and specimen processing methods are currently being used to measure HIF-1 α in early-phase clinical trials at the NCI of HIF-1 α -inhibiting anticancer agents. Our assay could also be applied to

investigate perturbations of HIF-1 α by drugs that indirectly affect the oxygenation status of tumors. For example, we have recently observed increased levels of HIF-1 α in xenografts after administration of pazopanib, a potent inhibitor of VEGFRs, platelet-derived growth factor receptors (PDGFRs), and c-kit tyrosine kinases, which might be explained by intra-tumoral hypoxia produced by this anti-angiogenic agent (data not shown) [36–38].

In conclusion, oxygen-sensitive HIF-1 α protein can be stabilized in tumor specimens using our specimen collection and processing procedures. We have shown that combining this sample handling method and a validated ELISA to quantify HIF-1 α levels in solid tumor tissues is useful for monitoring the pharmacodynamic effects of HIF-1 α inhibitors in preclinical samples. The clinical applicability of these methods was also demonstrated in patient tumor biopsies. We believe our ELISA will become a useful tool for measuring pharmacodynamic effects in preclinical modeling and clinical trials of novel HIF-1 α targeting agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

2-HG	2-hydroxyglutarate
ATCC	American Type Culture Collection
BCA	bicinchoninic acid
CV	coefficient of variation
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbant assay
ERK	extracellular signal-regulated kinase
H-CEB	HIF-1 α cell extraction buffer
HIF-1α	hypoxia-inducible factor-1 alpha
IP	intraperitoneally
IV	intravenously

LLQ	lower limit of quantitation
MAPK	mitogen-activated protein kinase
NCTVL	National Clinical Target Validation Laboratory
PADIS	Pharmacodynamic Assay Development and Implementation Section
PBS	phosphate buffered saline
PDGFR	platelet-derived growth factor receptor
PHD	prolyl hydroxylase
PI3K	phosphatidylinositol 3-kinase
QDxN	treated once daily for N days
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
VEGF	vascular endothelial growth factor

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Figure 1.

Method development and analytical validation of the HIF-1α ELISA. **A.** Standard curve plotted from the average of 6 plates of data with recombinant HIF-1α protein ranging from 7.18 to 1000 pg/mL. Values for the high, mid, and low control lysates analyzed on the same plates are also plotted. Data are mean $\pm$ SD. **B.** Dilution linearity was determined for HIF-1α read out values from HCT-116 xenograft extracts over increasing total protein loads ranging from 1.25 to 20 μg total protein. Extracts were assayed in triplicate and the mean of the triplicate wells were normalized to 1 μg total protein load per well; for all samples the 1.25 μg load was below the LLQ of the assay. **C.** Intra-assay or inter-assay precision for HIF-1α standards and assay controls. Standards and controls were run as unknowns and read against the standard curve in six plates on three separate days. Standards were assayed over the dynamic range of the assay (7.8–1000 pg/mL; filled symbols) and all three control levels (open symbols) were analyzed. **D.** Comparison of HIF-1α ELISA results from 18 matched samples, including assay controls, conducted by two independent laboratories, the National Clinical Target Validation Laboratory (NCTVL) and the Pharmacodynamic Assay Development and Implementation Section (PADIS). Sample dilution and analysis were performed independently by both laboratories and HIF-1α levels were compared across sites.

Figure 2.

Specimen processing methods influence the recovery of HIF-1α. **A**, Western blot analyses of HIF-1α protein expression in PC-3 cells grown and lysed under normoxic (N) or hypoxic (H) conditions. Cells were either lysed immediately or flash frozen and stored at -80°C for 1 day until lysis. 40 μg protein loads per well. **B**, Western blot analyses of HIF-1α protein expression in PC-3 cells cultured and lysed under normoxic (N) and hypoxic chamber (H) conditions similar to Panel A. The sample in lane 1 was flash frozen and lysed in normoxic conditions 1 day (N 1d) after storage at -80°C , the sample in lane 2 was lysed immediately

in a hypoxic chamber, and samples in lanes 3 and 4 were flash frozen in a hypoxic chamber and then lysed in normoxic conditions 1 hour (N 1h) or 5 days (N 5d) after storage at -80°C . This figure demonstrates that the most critical step in recovery of HIF1 α is rapid collection and flash-freezing of the specimen under hypoxic conditions; use of degassed lysis buffer (H-CEB) to thaw and lyse the cultured cells is sufficient to preserve HIF1 α when the cells properly collected.

C, Quantitative ELISA performed on extracts from SiHa and PC3 cells grown under hypoxic conditions with 1 μM bortezomib and then lysed by sonication in normoxic conditions with degassed H-CEB containing 100 μM bortezomib ($n = 3-6$ flasks/time point).

D, Quantitative ELISA readout of HIF-1 α levels in PC-3 cells grown under normoxic or hypoxic conditions and then lysed in normoxic conditions by either tissue grinding or sonication. **E**, Quantitative ELISA readout of HIF-1 α levels in matched HCT-116 xenograft quadrants ($n = 6$ animals) lysed using different tissue homogenization methods. Each quadrant from a xenograft was flash-frozen, lysed in degassed buffer, and then homogenized by either sonication or with Precellys bead homogenization using 1.4-mm ceramic, 2.8-mm ceramic, or 2.8-mm metal beads. For each sample, 1.25 to 10 μg total protein was loaded per well. Data are mean $\pm$ SD. Asterisk (*), $P < 0.05$ when compared with any of the bead homogenized samples (paired t -test, 2-tailed). **F**, HIF-1 α levels measured in SiHa cell lysates incubated with 1 μM bortezomib for 4 hours then lysed in degassed H-CEB alone or containing 100 μM (R)- or (S)-2-HG from Sigma-Aldrich (SA) or (R)-2-HG from 3BSC (lot# WH20120516).

Figure 3.

ELISA readout of HIF-1α levels in different collection methods, lysis buffers, and biopsy depths. **A**, HIF-1α in needle biopsy samples from DU145 xenografts from untreated mice collected by two different methods: flash-freezing after collection in nitrogen gas-purged H-CEB or dry flash-freezing. Mice were randomly divided into 2 groups, 24 mice per group. Specimen collection in degassed H-CEB followed by flash-freezing significantly increased HIF-1α protein levels compared to dry flash-freezing (*unpaired *t*-test, 2-tailed, $P = 0.004$). **B**, HIF-1α levels in shallow versus deep biopsies collected from the same animal. **C**, HIF-1α levels in SiHa cell lysates incubated with 1 μM bortezomib for 4 hours then lysed in degassed H-CEB alone or containing 100μM bortezomib, MG-132, or 2-HG (3BSC, lot# WH20100114); *unpaired *t*-test, 2-tailed, $P < 0.05$. **D**, HIF-1α levels in lysates of A375 xenografts from vehicle-treated mice ($n = 4$). Each xenograft tumor was divided into 4

quadrants and lysed in H-CEB alone, with 100 μ M bortezomib, with 2-HG, or with both agents (*paired *t*-test, 2-tailed, $P = 0.009$). Data are mean $\pm$ SD.

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Figure 4.

Pharmacodynamic response to HIF-1 α -inhibiting drugs in in vivo models. **A**, HIF-1 α levels in A375 xenografts from mice treated with vehicle for QDx5; with NSC 743400 at 25 mg/kg (MKG) for QDx1; with NSC 743400 at 25, 12.5, or 2.5 MKG for QDx5, or with topotecan at 4 MKG for QDx5 (n = 3 animals per group). Whole xenografts were surgically excised 1 hour after the last dose and one quadrant was processed. **B**, HIF-1 α levels in PC-3 xenografts from mice treated with vehicle or 4 MKG topotecan for QDx2 (n = 4 and 8, respectively); or with vehicle, 4 MKG, or 1.5 MKG topotecan for QDx5 (n = 4, 8, and 8,

respectively). Whole xenografts were surgically excised 7 hours after the last dose; one quadrant was processed. **C**, HIF-1 α levels in HCT-116 xenografts from mice treated with a single dose of vehicle or selumetinib (50 MKG). Needle biopsies (18-g) were collected 2, 4, 7, and 24 hours after dosing, 6 animals per treatment group. **A–C**, Values represent mean $\pm$ SD. *, $P < 0.05$ when compared with the controls (unpaired t -test, 2-tailed).

Table 1Spike recovery of HIF-1 α standards in HCT-116 xenograft extracts

HIF-1 α standard (pg/mL)	% Recovery by protein load							
	Xenograft 1				Xenograft 2			
	2.5 μ g	5 μ g	10 μ g	10 μ g	2.5 μ g	5 μ g	10 μ g	10 μ g
31.25	99.4	97.4	94.1	94.1	105.2	100.3	110.7	110.7
62.5	96.5	98.2	95.4	95.4	99.8	110.7	117.6	117.6
125	96.1	96.2	94.2	94.2	114.5	128.4	104.5	104.5
250	95.6	95.2	91.4	91.4	109.2	105.5	94.2	94.2
500	96.6	96.0	89.5	89.5	95.8	95.2	91.0	91.0
1000	98.3	96.3	90.1	90.1	101.3	99.1	93.3	93.3
Overall recovery (mean $\pm$ SD)	95.4 $\pm$ 2.7				104.2 $\pm$ 9.7			

Table 2

HIF-1 α levels in PC-3 cells grown under either normoxic or hypoxic conditions and homogenized by sonication.

	Average HIF-1 α (pg/ μ g protein)	Average Actin (ng/ μ g protein)	HIF-1 α (pg)/ actin (ng)	HIF-1 α molecules/cell
Normoxic	6.41	9.15	0.70	926
Hypoxic	23.86	10.44	2.29	2856

HIF-1 α protein levels in 18-gauge needle tumor biopsies from patients with advanced refractory disease enrolled in clinical trials at the NCI

Table 3

Patient	Tumor type	Biopsy Site	Months at -80°C ^a	Protein lysate (μg)	HIF-1 α Read out <i>b</i> (pg/μg protein)
1	Colon cancer	Liver	19.6	1048	1.28 ± 0.06
2	Neuroendocrine tumor	Liver	18.2	378	0.32 ± 0.01
3	Breast cancer	Liver	2.8	355	0.27 ± 0.02
4	Colon cancer	Liver	2.3	450	0.20 ± 0.02
5	Alveolar soft part sarcoma	Thigh mass	7.2	308	0.10 ± 0.01
6	Hepatocellular carcinoma	Liver	13	430	0.39 ± 0.02
7	Melanoma	subcutaneous nodule	7.9	590	0.38 ± 0.01

^aTotal number of months that specimens were stored, flash-frozen, at -80°C under normoxic conditions. Specimens were thawed into degassed H-CEB for analysis per the described immunoassay methods.

^bLysates of each sample were loaded at three different protein loads with H-CEB, in triplicate, and the HIF-1 α levels were averaged and back-calculated based on μg total protein loaded. The data shown are mean ± standard deviation.