Original Articles

The BH3-mimetic obatoclax reduces HIF-1α levels and HIF-1 transcriptional activity and sensitizes hypoxic colon adenocarcinoma cells to 5-fluorouracil

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Abstract

Activation of hypoxia-inducible factor (HIF)-1 is a feature of hypoxic solid tumors that has been associated with drug resistance, mainly due to disruption of Bcl-2 family dynamics. Resetting the balance in favor of proapoptotic family members is an attractive therapeutic goal that has been pursued by developing BH3-mimetic compounds. In the present study we evaluated the response of human colon adenocarcinoma cells to the BH3-mimetic obatoclax (OBX), in terms of growth arrest, apoptosis and autophagy, in the presence or absence of HIF-1α-stabilizing conditions; its possible effect on HIF-1α expression and HIF-1 activity; and the possibility to improve the response of colon cancer cells to cytotoxic chemotherapy by combining them with OBX. Colon cancer cell response to the BH3-mimetic was unmodified by HIF-1 activation and OBX induced a decrease in HIF-1α protein levels and HIF-1 transcriptional activity, probably by decreasing HIF-1α synthesis and facilitating a VHL-independent proteasomal degradation pathway. Finally, a chemosensitizing effect of OBX with respect to 5-fluorouracil or oxaliplatin treatment was observed, highlighting the possibility that patients with hypoxic colon tumors might benefit from combined regimens including OBX.

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Introduction

Solid tumor growth easily outstrips oxygen and nutrient supply through pre-existing and newly formed vessels. Thus, hypoxia is a common feature in solid tumors that has emerged as a powerful driving force for tumor progression and a major cause for therapeutic failure [1,2]. Adaptation to hypoxia depends on activation of a family of heterodimeric transcription factors, the hypoxia-inducible factors (HIFs), that accumulate in the nucleus following oxygen-dependent stabilization of the inducible α subunit. Under normoxic conditions this is continuously synthesized and degraded following hydroxylation of two critical proline residues (P402 and P564) by specific prolyl hydroxylases (PHD1-3), and polyubiquitylation mediated by the Von Hippel-Lindau protein (pVHL) [3]. HIFs modulate key aspects of tumor biology, including angiogenesis and metastasis, cell survival, resistance to apoptosis and metabolic reprogramming [4]. HIF-1 has been implicated in anticancer drug resistance in a variety of tumor models, including colon cancer [5–8], by inducing multidrug efflux transporter expression [9,10], interfering with DNA repair [11] and, most notably, repressing apoptosis by shifting the balance between pro- and anti-apoptotic Bcl-2 family members [12–15].

The role of the Bcl-2 family in the resistance to both conventional cytotoxic agents and newer targeted therapeutics has been extensively demonstrated [16]; thus, a novel class of small molecule inhibitors of the pro-survival members of the Bcl-2 family, dubbed BH3-mimetics, has been developed with the aim to directly activate the apoptotic machinery, induce cell death and/or restore drug sensitivity [17,18]. One such agent is obatoclax (OBX), a synthetic indolylprodigiosin derivative that was shown to bind to Bcl-2, Bcl-xl, Bcl-w and Mcl-1 [19–21]. Preclinical studies have shown that OBX is effective as a single agent against a broad range of cancer cell lines and xenografts [22] and that it synergizes with a number of cytotoxic and targeted anticancer agents [23–29]. Clinical activity and tolerability have been assessed in a number of phase I and II trials (clinicaltrials.gov).

The ability of OBX to reset the proapoptotic/pro-survival balance within the Bcl-2 family suggests that it could retain its activity under hypoxic conditions. Thus, in the present study we evaluated the
response of human colon adenocarcinoma cells, in terms of growth arrest, apoptosis and autophagy, to OBX, under control and HIF-1α-stabilizing conditions, and its possible effect on HIF-1α expression and HIF-1 activity. Our results indicate that cell response to OBX is unaffected by HIF-1 activation and that the BH3-mimetic compound down-regulates both HIF-1α expression and HIF-1 activity; moreover, OBX sensitizes hypoxic colon cancer cells to the action of 5-fluorouracil (5-FU) and oxaliplatin (oxPt), suggesting that patients with hypoxic tumors might derive a therapeutic benefit from combined regimens including OBX.

Materials and methods

Standard chemicals and cell culture reagents, unless otherwise indicated, were purchased from Sigma Aldrich.

Cell lines

Human colon cancer cell lines HCT116, HCT-8, HT29 were originally obtained from the American Type Culture Collection and recently authenticated by morphological inspection, growth curve analysis and short tandem repeat profiling. All cell lines were maintained in DMEM (Dulbecco’s Modified Eagle’s Medium), supplemented with 10% fetal bovine serum, 2% glutamine, 1% non-essential amino acids at 37 °C in a humidified 5% CO2 atmosphere and were routinely checked for Mycoplasma (Molecular Biology Reagent Set Mycoplasma species, Euroclone).

Hypoxia and HIF-1α stabilization

Hypoxia was induced by placing the cells for 24 h into a modular incubator chamber (Billups Rothenberg Inc., Del Mar, CA, USA) flushed with a mixture of 1% O2, 5% CO2 and 94% N2 at 37 °C. To achieve oxygen-independent HIF-1α stabilization, cells were exposed to 100 μM CoCl2 for 24 h, a condition that inhibits hydroxylation of p402 and P564 and the subsequent interaction of HIF-1α with pVHL, thereby stabilizing HIF-1α [30].

Cytotoxicity studies and drug treatment

Cell survival following exposure to OBX (10–200 nM, Selleck Chemicals LLC) and/or 5-FU (2.5–75 μM) or oxPt (0.1–5 μM) was evaluated using the MTT assay, as described elsewhere [6,31]. Cells were treated with 5-FU or oxPt and/or OBX for 48 h and exposed to hypoxia or CoCl2 for the last 24 h. The OBX concentration range was chosen to include peak concentrations achievable in patients without intolerable adverse events [32]. To understand the role of the autophagic process in the response of HCT116 cells to the 5-FU/OBX combination, MTT assays were also performed in the presence of the autophagy inhibitor bafilomycin A1 (BafA1, 1 nM). Cell viability was determined by measuring the 570 nM absorbance in each well, using a Universal Microplate Reader EL800 (BioTek Instruments).

Assessment of HIF-1α and HIF-2α expression

HIF-1α and HIF-2α protein levels were detected by Western blot (WB) analysis of whole cell lysates obtained from control, hypoxic and CoCl2-treated HCT116 cells, following treatment with OBX (25 and 50 nM) as described above. Cell extracts were prepared as detailed elsewhere [6]. To investigate the effects of OBX on HIF-1α degradation, during the last 4 h of CoCl2/OBX treatment the time course of HIF-1α disappearance was monitored in the presence of the protein synthesis inhibitor cycloheximide (CHX, 100 μM), harvesting the cells at different time points (0–15–30–60–120 and 240 min). Conversely, the effect of OBX on HIF-1α synthesis was assessed by exposing the cells to the proteasome inhibitor MG132 (10 μM) during the last 4 h of CoCl2/OBX treatment. Protein concentration was determined by the BCA assay (Pierce, Italy); 50 μg of protein per sample were loaded onto polyacrylamide gels (7%) and separated under denaturing conditions. Protein bands were transferred onto PVDF Hybond-P membranes (Amersham Biosciences) and WB analysis was performed by standard techniques with mouse monoclonal anti-human HIF-1α antibody (BD Biosciences) or rabbit polyclonal anti-human HIF-2α antibody (Novus Biologicals); β-tubulin served as loading control, using a mouse monoclonal antibody (Sigma Aldrich). Protein bands were visualized using a peroxidase-conjugated anti-mouse (HIF-1α and β-tubulin) or anti-rabbit secondary antibody (HIF-2α) and the Supersignal West Femto Maximum Sensitivity Substrate (Pierce). WB was also used to assess Bcl-2 family protein levels (Appendix S1, Supplementary Methods).

Vectors and transfection

HCT116/HRP-EGFP cells were obtained by transfecting HCT116 cells with a plasmid containing the EGFP cDNA under the control of an artificial hypoxia-responsive promoter (HRP) consisting of five copies of a 35-bp fragment from the Hypoxia Response Element (HRE) of the human VEGF gene and a human cytomegalovirus (CMV) minimal promoter (kindly provided by Dr. Y. Cao, Duke University, NC, USA [33]). HCT116 cells were seeded onto 6-well plates (2.5×105 cells/well) and allowed to attach for 24 h before lipofection using Lipofectamine® 2000 (Invitrogen). EGFP expression in transfected cells (following HIF-1α stabilization with CoCl2 at 100 μM for 24 h) persisted for 6–8 days; all the experiments were performed within 5 days of transfection.

HCT116/NDHIF-1α cells were obtained by transfecting HCT116 cells with a plasmid containing a 3100 bp fragment encoding a mutant form of the HIF-1α gene (P402A and P564R) that cannot undergo pVHL-mediated degradation [34] (kindly provided by Dr. Chris Paraskeva, Bristol University, UK) or with an empty pCDNA3 vector.

Flow cytometric analysis

Cell cycle distribution and percentage of apoptotic cells in HCT116 cells and HIF-1α transcriptional activity in HCT116/EGFP-HRP cells, were assessed using a FACScan (Becton Dickinson), equipped with a 15 mW, 488 nm and an air-cooled argon ion laser. EGF (Enhanced Green Fluorescent Protein) fluorescent emissions were collected from freshly harvested cells, in log mode, in the FL1 channel (530/30); fluorescence intensity was expressed as mean fluorescence channel (MFC). Samples for apoptosis and cell cycle analysis were fixed in 70% ethanol at −20 °C and incubated with propidium iodide (50 μg/ml) and RNAse (20 μg/ml) for 30 min in the dark. Fluorescent emissions were collected through a 575 nm band-pass filter; the percentage of apoptotic cells was determined based on sub-G1 peaks in monoparametric histograms.

Assessment of autophagy

Induction of autophagy by OBX, as a single agent or in combination with 5-FU or oxPt, was assessed based on WB of total cell extracts, using a rabbit polyclonal antibody directed against the autophagosomal marker LC3-II (Cell Signaling).

Quantitative RT-PCR

The transcript levels of the HIF-1α and of the HIF-1 target gene IGF2 were analyzed in OBX-treated cells incubated with CoCl2 for the last 24 h. Total RNA was extracted using the NucleoSpin® TriPrep Kit (Macherey-Nagel) following the manufacturer’s instructions, and quantitated (ND-1000, NanoDrop, Thermos Fisher Scientific); 2 μg of RNA were retro-transcribed (High Capacity cDNA Reverse Transcript Kit, Applied Biosystem) and 50 ng (5 μl) of the resulting cDNA were added with 15 μl of a solution containing 5× Master Mix (TaqMan, Applied Biosystems), 20× reverse and forward primers (Applied Biosystems) and MilliQ H2O. The run was performed in a Thermocycler Chromo 4TM Real-Time PCR Detection System (BIO-RAD), following the protocol provided by the manufacturer and the results analyzed by the Optionn Monitor 3 software (BIO-RAD).

Statistical analysis

In IC50 Values, percentage of apoptotic cells, cell cycle distribution, HIF-1α and IGF2 transcript levels, densitometric measures of HIF-1α protein levels and HIF-1-dependent transcriptional activity in HCT116/EGFP-HRP cells with or without OBX and/or CoCl2 were evaluated by two-way ANOVA. Survival data following OBX treatment under control, hypoxic conditions and following CoCl2 incubation were analyzed by non-linear regression analysis, fitting a sigmoid dose–response curve model; non-linear regression analysis was also used to evaluate differences in the time course of HIF-1α degradation, fitting a one-phase exponential decay model. All statistical analyses were performed using the Prism 5.0 software (GraphPad Software, Inc.)

Results

Obatoclax retains its antiproliferative and proapoptotic effects under hypoxia and HIF-1α-stabilizing conditions

Fig. 1A shows the concentration/response curves obtained in HCT116 cells following 48 h exposure to OBX under control conditions or in the presence of hypoxia or 100 μM CoCl2 during the last 24 h. The results indicate that the effect of OBX was unmodified by conditions preventing HIF-1α hydroxylation and VHL-dependent degradation. Similar results were also obtained for two other colorectal cancer cell lines, HCT-8 and HT29 (Table 1). Under the same experimental conditions neither hypoxia nor CoCl2 exposure caused a decrease in the proapoptotic effect of OBX, but resulted
in fact in a significant increase in apoptotic cells (Fig. 1B). Cell cycle analysis of OBX treated cells (Fig. 1C) shows that OBX caused a moderate but significant increase in G1 cells and a corresponding decrease in G2/M cells; HIF-1α stabilization did not affect this behavior. OBX also increased LC3-II levels in HCT116 cells under both experimental conditions (Fig. 1D); addition of BaA1 to the OBX treated cells resulted in decreased cell survival, irrespective of HIF-1α stabilization (Fig. 1E).
Obatoclax induces a decrease in HIF-1α protein levels and HIF-1 transcriptional activity

The assessment of Bcl-2 family protein levels following OBX treatment of HCT116 cells indicates that OBX did not substantially modify the levels of pro- and anti-apoptotic proteins, while decreasing the inhibitory association between Mcl-1 and Bax, according to its BH3-mimetic action; the presence of CoCl₂ did not modify this effect (Appendix S1, Supplementary methods, and Supplementary Fig. S1).

Fig. 2A shows a typical western blot of HIF-1α and HIF-2α protein levels, along with densitometric analysis of three independent experiments. In hypoxic and CoCl₂-treated cells OBX caused a significant concentration-dependent decrease in HIF-1α protein levels (that are undetectable in control samples); in contrast, HIF-2α expression was observed under control conditions, albeit at lower levels with respect to hypoxia or CoCl₂ exposure, and was unaffected by OBX under any of the experimental conditions tested. Interestingly, a comparable significant decrease in HIF-1α protein levels was also observed when OBX was added to normoxic HCT116/NDHIF-1α expressing a mutant form of HIF-1α that cannot be prolyl hydroxylated (Fig. 3). This suggests that the canonical (“normoxic”) VHL-dependent route degradation is not involved in OBX-induced decrease in HIF-1α levels, as already indicated by the results obtained in CoCl₂-treated cells.

To verify that the decrease in HIF-1α protein levels corresponded to a decrease in HIF-1 transcriptional activity, we evaluated the effect of OBX on the fluorescence emitted by HCT116/pEGFP HRP cells, in which EGFP expression is under the control of an artificial hypoxia-responsive promoter (HRP); as a positive control, we used HCT116/pEGFP N1 cells, constitutively expressing EGFP. No fluorescent signals were obtained from HCT116 mock-transfected cells, used as negative controls (data not shown). Fig. 4A shows that, as expected, both control and CoCl₂-treated HCT116/pEGFP N1 cells exhibited high constitutive levels of fluorescence that were not modified by OBX. In contrast, in HCT116/pEGFP HRP CoCl₂ exposure induced a significant increase in fluorescence that reverted to control values following exposure to both OBX concentrations used, indicating that the decrease in HIF-1α levels induced by OBX was in fact associated with a significant decrease in HIF-1 transcriptional activity. As a further support, RT-PCR analysis of IGF2 mRNA levels (IGF2 being a well known HIF-1 target gene [35]) showed a significant increase following CoCl₂ exposure, whereas control levels were restored in CoCl₂/OBX-treated cells (Fig. 4B).

Obatoclax modulates HIF-1α protein levels by post-transcriptional mechanisms

As shown in Fig. 5A, no significant differences in HIF-1α transcript levels were observed between control and CoCl₂-treated cells, and treatment with OBX did not cause any change, thus ruling out a possible effect of the compound at the transcriptional level.

To evaluate whether OBX affected the rate of HIF-1α degradation via VHL-independent mechanisms, CoCl₂/OBX-treated cells were

<table>
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<th>Control</th>
<th>pO₂ 1%</th>
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<tr>
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<td>71.4 ± 5.57</td>
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<td>31.7 ± 2.84</td>
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<tr>
<td>HCT-8</td>
<td>76.0 ± 7.75</td>
<td>80.8 ± 8.04</td>
<td>74.4 ± 3.12</td>
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Table 1
IC₅₀ values obtained from dose–response curves to OBX in HCT116, HT29 and HCT-8 cells following 48-h treatment with OBX (10–200 nM) and incubation in normoxia, hypoxia or in the presence of 100 μM CoCl₂ for the last 24 h (means ± s.e.m. of 3–5 independent experiments).
harvested at different time points (0–15–30–60–120 and 240 minutes) after the addition of the protein synthesis inhibitor CHX (100 μM). HIF-1α levels (Fig. 5B) were determined by densitometric analysis of Western blots, and the rate of HIF-1α decrease was compared with that observed in OBX-untreated counterparts (Fig. 5C). Two-way ANOVA of the results indicated a significant difference between the curves obtained in the presence or absence of OBX, starting at 30 min after CHX addition and up to the 240 min time point; non-linear regression analysis of the curves yielded approximate HIF-1α half-life values of 135 min in untreated cells and 60 min in OBX-treated cells. Conversely, to examine whether OBX affected HIF-1α synthesis in CoCl2-treated cells, during the last 4 h of the experiment we added the proteasome inhibitor MG132 (10 μM), thus effectively blocking VHL-independent pathways for HIF-1α proteasomal degradation. Fig. 5D shows that OBX retains its ability to decrease HIF-1α protein levels in the presence of MG132, thereby indicating a probable effect on protein synthesis.

Obatoclax potentiates the effect of chemotherapy under HIF-1α-stabilizing conditions

Based on the newly discovered ability of OBX to down-regulate HIF-1α levels, we investigated whether the compound could be used to potentiate the cytotoxic effect of 5-FU, under HIF-1α-stabilizing conditions. Fig. 6A shows the dose–response curves obtained in HCT116 cells following 48 h exposure to 5-FU, with or without 50 nM OBX, under control conditions or in the presence of 100 μM CoCl2 during the last 24 h. 5-FU cytotoxicity was significantly diminished in the presence of CoCl2, confirming earlier observations by our group [6]; at the selected concentration, OBX exhibited minimal toxicity as a single agent and did not significantly modify the response to 5-FU under control conditions. However, simultaneous exposure to 5-FU and 50 nM OBX caused the IC50 value of 5-FU to revert to the control values observed in the absence of CoCl2. Similar results were obtained in HCT8 cells (Supplementary Fig. S2). Fig. 6B shows that stabilization of HIF-1α decreased the percentage of apoptotic cells following 5-FU exposure, whereas addition of OBX increased apoptosis over the levels observed with 5-FU alone, irrespective of HIF-1α stabilization. In addition, OBX also increased LC3-II levels in 5-FU treated cells under both experimental conditions (Fig. 6C, densitometric analysis in Supplementary Fig. S3). Similar results, in terms of survival curves, apoptotic cell death and LC3-II accumulation, were observed with the oxPt/OBX combination in HCT116 cells (Supplementary Fig. S4). Finally, Fig. 6D shows that addition of BaA1 to the 5-FU/OBX combination resulted in decreased cell survival, irrespective of HIF-1α stabilization.

Discussion

The search for specific HIF-1 inhibitors has been very active for the past few years, in view of its documented role in tumor progression and drug resistance [1,2], but serious clinical candidates have yet to emerge. In addition, both HIF-1-dependent and -independent mechanisms have been implicated in hypoxia-induced drug resistance, and specifically in the down-regulation of proapoptotic proteins [15,36]; thus, HIF-1 inhibitors would probably not be sufficient to effectively overcome hypoxia-induced resistance. A viable complementary strategy could involve the use of BH3-mimetic compounds, a novel class of small molecule targeted agents that mimic BH3-only proapoptotic proteins by
interacting with the hydrophobic groove of pro-survival Bcl-2 proteins, thereby inhibiting their function. These agents have shown a good safety profile in early clinical trials and several phase II trials are ongoing at the time of this writing (clinicaltrials.gov).

BH3-mimetics appear to be uniquely suited to counteract the effects of hypoxia on Bcl-2 proteins, provided that their activity remained unaffected by the adaptive hypoxic response. Two recent studies by the Dive group provided encouraging evidence in this regard, showing that the BH3-mimetic ABT-737 not only retains its efficacy in hypoxic cancer cells, but is actually more effective than in their normoxic counterparts [37,38]. In the present study we focused on OBX, that differs from ABT-737 for a number of features, including a broader specificity across the panel of pro-survival Bcl-2 proteins, with which it can interfere both directly and indirectly [39,40], and the ability to elicit a spectrum of “off-target” effects [41,42] that might contribute to its antitumor activity. Most interestingly, OBX has shown the ability, so far unique among BH3-mimetics, to inhibit mTOR kinase activity at both functionally active complexes, mTORC1 and mTORC2 [43], thereby preventing full Akt activation and undermining the prosurvival role of the PI3K–Akt–mTOR pathway. In addition, OBX has been shown to induce autophagy as well as apoptosis [42]. While autophagy is known to play different, context-dependent roles in drug response, its induction by chemotherapeutic agents has mostly been described as a cytoprotective response. Thus, the simultaneous triggering of a cytoprotective process by OBX might overshadow the impact of apoptosis on overall cell survival. Accordingly, the autophagic inhibitors 3-methyladenine and chloroquine have been shown to increase the cytotoxic effect of OBX in esophageal carcinoma cells [25]; however, recent evidence indicates that in a different experimental model (breast cancer cells) OBX induces a form of toxic autophagy by obstructing autophagic flux and inhibiting autophagic degradation of damaged cellular protein components, thus causing cell death mediated by endoplasmic reticulum stress [44].

Our initial experiments in the present study confirmed that IC50 values obtained for OBX by the standard MTT assay were unaffected in three different colon cancer cell lines by incubation either at 1% pO2 or in the presence of the hypoxia mimetic CoCl2 (Fig. 1A and Table 1), two experimental conditions that prevent HIF-1α degradation through the canonical “normoxic” (PHD/VHL/ubiquitin-dependent) pathway. In HCT116 cells, besides the expected pro-apoptotic effect (Fig. 1B), OBX was also found to cause cell cycle arrest (Fig. 1C), as described by Koehler et al. in other colon cancer cell lines [45]; the fact that, at variance with the results of the present study, these Authors reported cell cycle arrest in G2/M rather than in G1 could depend on cell type-specific differences and/or on the

Fig. 5. OBX modulates HIF-1α expression at a post-transcriptional level. (A) HIF-1α transcript levels in HCT116 cells following OBX treatment (25 and 50 nM for 48 h), in the presence of 100 μM CoCl2 during the last 24 h; data are the means ± s.e.m. of 3 independent experiments. (B) Effect of 48 h treatment with 50 nM OBX, in the presence of 100 μM CoCl2 (for the last 24 h) and of the protein synthesis inhibitor CHX (100 μM) during the last 4 h of treatment, on the decay of HIF-1α protein levels in HCT116 cells. (C) Densitometric analysis of the immunoreactive bands from 3 independent experiments performed as in panel B. The means ± s.e.m are reported following normalization vs. β-tubulin levels; C, control; OBX: p < 0.05, p < 0.001 and p < 0.001 vs. control by two-way ANOVA and Bonferroni t test. (D) HIF-1α protein levels in HCT116 cells following treatment with 50 nM OBX for 48 h in the presence of 100 μM CoCl2 (for the last 24 h) and the proteasome inhibitor MG132 (10 μM during the last 4 h); densitometric analysis following normalization vs. β-tubulin levels is also shown (means ± s.e.m. of 3 independent experiments).
fact that Koehler et al., by design, used subcytotoxic concentrations of OBX.

Interestingly, while survival curves obtained for OBX under the three experimental conditions tested were totally superimposed in our preliminary experiments, the percentage of apoptotic cells induced by OBX increased significantly following hypoxic or chemical HIF-1α stabilization in HCT116 cells (Fig. 1B). This apparent paradox might be due to a counterbalancing effect of unspecified hypoxia-related factors, such as the release of IGF2, which would replenish the cell population by stimulating cell proliferation; however, while the observed increase in IGF2 expression in CoCl2-treated HCT116 cells (Fig. 4B) would appear to support this hypothesis, the results of cell cycle analysis strongly argue against it. An alternative explanation, as mentioned above, might involve the ability of OBX to trigger cytoprotective autophagy. OBX was found in fact to induce autophagy, irrespective of HIF-1α stabilization (Fig. 1D); blocking autophagy with BaA1 was shown to further decrease cell survival (Fig. 1E).

Overall, the results of our preliminary experiments indicate that preventing HIF-1α degradation does not significantly modify tumor cell response to OBX, in terms of global cell survival and of induction of apoptosis and autophagy. The novel finding in the present study is that OBX was actually able to reduce HIF-1α accumulation, in spite of the stabilizing conditions adopted in our experiments. Fig. 2A shows a concentration-dependent decrease in HIF-1α protein levels in whole protein extracts obtained from hypoxic and CoCl2-treated cells. Increased degradation through the canonical pathway can be ruled out under conditions where O2 is depleted or PHD activity is directly inhibited by CoCl2 [30]; and the observation that HIF-2α (undergoing the same normoxic degradation pathway)
protein levels were unaffected by OBX indicates that the block was still operating. Additional evidence comes from the observation that OBX also caused a decrease in HIF-1α levels in HCT116/NDHIF-1α cells, expressing a mutant form of HIF-1α, devoid of the 2 critical proline residues that are necessary for interaction with VHL. (Fig. 3).

As a consequence of the decrease in HIF-1α protein levels induced by OBX, a significant decrease in HIF-1 transcriptional activity was also observed. This is apparent from the reduced fluorescence in OBX-treated cells in the GFP-based transcriptional reporter assay illustrated in Fig. 4A, and from the concentration-dependent decrease in the HIF-1 target gene IG2 transcripts (Fig. 4B).

In our endeavor to explain the mechanism(s) of the OBX-induced decrease in HIF-1α protein levels we found that mRNA levels were unmodified, indicating that post-transcriptional mechanisms are probably involved. Upon blocking HIF-1α synthesis with CHX (Fig. 5B), we observed that, in spite of the CoCl2-induced block of prolyl hydroxylation-dependent proteasomal degradation, HIF-1α levels decreased throughout the 48 h experimental period. This is in agreement with the findings of Kong et al. [46], indicating that HIF-1α is slowly but continuously down-regulated in hypoxia through a proteasome-dependent, but O2/VHL/ubiquitin-independent, mechanism. Analysis of the time course of HIF-1α degradation indicates that in the presence of 50 nM OBX the process was accelerated, supporting a role for facilitated HIF-1α degradation. When cells were exposed to the proteasome inhibitor MG132 (Fig. 5D), a marked increase in HIF-1α protein levels could be observed, further confirming that the PDH/VHL/ubiquitin pathway blocked by CoCl2 (and O2 depletion) is by no means the only proteasome-dependent pathway for HIF-1α degradation [46]. Our results show that, when all proteasome-dependent pathways are inhibited, OBX still retains its ability to decrease HIF-1α protein levels, possibly by inhibiting its synthesis. The failure to observe a similar decrease in HIF-2α levels following OBX exposure seems to rule out a generalized effect on protein synthesis. The already mentioned ability of OBX to inhibit mTORC1 and mTORC2 in melanoma cells provides a clue to one possible mechanism for OBX-induced decrease in HIF-1α protein synthesis [43,47].

Based on these results, we hypothesized that combining OBX with cytotoxic chemotherapeutics might achieve a dual goal in defeating hypoxia-induced drug resistance: 1) OBX might sensitize tumor cells in which apoptosis is impaired (whether by HIF-1-dependent mechanisms or otherwise) by directly activating the mitochondrial apoptotic machinery. The results of the present study confirm that, while OBX did not significantly modify Bcl-2 family expression levels, it did indeed interfere with the Mcl-1/Bax association, irrespective of HIF-1α stabilization (Supplementary Fig. S1), which could facilitate mitochondrial events involved in apoptosis; 2) OBX-induced decrease in HIF-1 activity might slow down the acquisition of further malignant traits and/or determinants of drug resistance. While these two goals have only been partially investigated in the present study, the results obtained seem to support our working hypothesis. Combining OBX with 5-FU or oxPt did in fact potentiate the cytotoxicity of the two agents in HCT116, and this response was particularly significant under HIF-1α-stabilizing conditions (Fig. 6 and Supplementary Fig. S4). Blocking autophagy was shown to increase the effect of the 5-FU/OBX combination in HCT116 cells, suggesting that (a) as already observed for OBX alone, autophagy plays a cytoreductive role following exposure to the 5FU/OBX combination; and (b) blocking autophagy might increase the clinical benefit deriving from the use of OBX in combination with chemotherapy.

In summary, our study has highlighted the possibility that the response of hypoxic colon tumors to chemotherapy might be significantly improved by combination with OBX. Further investigations are in progress to extend our findings to other cell lines derived from different tumor types and, most importantly, to the in vivo settings.


