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Effect of HIF-1 modulation on the response of two- and three-dimensional cultures of human colon cancer cells to 5-fluorouracil

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ABSTRACT

Tumour hypoxia represents a major obstacle to the success of radiotherapy and chemotherapy. The discovery that the hypoxia-inducible factor 1 (HIF-1) is a master regulator of cellular response to low oxygen led to the concept that inhibiting HIF-1 activity may sensitise hypoxic cancer cells to radiation and cytotoxic drugs. In the present study we investigate the effects of HIF-1 modulation on the response of the human colon adenocarcinoma cell line HCT116 to 5-fluorouracil (5FU). Increasing HIF-1 activity, either by exposing cells to hypoxia or by forced expression of a degradation-resistant form of HIF-1α, results in poor cell response to 5FU; conversely, knockdown of HIF-1α by RNA interference prevents hypoxia-induced resistance to 5FU. PMX290, a thioredoxin-1 inhibitor, significantly inhibits HIF-1 activity and concomitantly sensitises hypoxic cells to 5FU. These results were confirmed in HCT116 cells grown as three-dimensional spheroids, a model that more closely reproduces the hypoxic environment of solid tumours.

1. Introduction

Solid tumours invariably develop hypoxic areas as actively proliferating cells rapidly outgrow oxygen and nutrient supply provided by existing vessels. Tumour hypoxia is a powerful driving force for malignant progression and an adverse prognostic factor in cancer patients.[1,2] Clinical and preclinical studies have firmly established that hypoxia is associated with impaired response to both radiotherapy and chemotherapy.[3] This latter effect is due in part to poor perfusion and restricted drug access to hypoxic areas[4]; however, a major role is played by activation of a family of hypoxia-inducible transcription factors (HIFs), orchestrating a coordinated adaptive response.[5,6] HIFs act as heterodimers, consisting of an oxygen-dependent α and a constitutively expressed β subunit. The mechanism underlying the oxygen-dependent regulation of the α subunit protein levels has been described in detail for the best characterised factor in the family, known as HIF-1. In the presence of oxygen, two conserved proline residues (Pro 402 and Pro 564) in the α subunit undergo oxygen-dependent hydroxylation; this modification allows HIF-1α to become ubiquitylated, which targets the factor for degradation by the 26S proteasome within minutes of its synthesis. Under hypoxic conditions, however, prolyl hydroxylation cannot take place; thus, HIF-1α can accumulate and translocate to the nucleus, where it dimerises with HIF-1β and binds hypoxia response elements (HREs) throughout the genome.[5]

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HIF-1 has been shown to regulate the expression of more than 100 genes encoding key factors in cell proliferation and survival, glucose metabolism, invasion and angiogenesis.[5] Regarding tumour response to drug treatment, HIF-1 upregulation has been shown to induce expression of drug efflux transporters,[7,8] to alter the activity of DNA repair mechanisms[9] and to shift the balance between pro- and antiapoptotic factors towards cell survival,[10–12] thereby decreasing the effectiveness of a number of currently used anticancer agents.

Based on these observations, in recent years considerable efforts have focused on HIF-1 modulation as a strategy to inhibit tumour growth and metastasis, as well as to sensitise tumours to chemotherapy. A number of small molecule and nucleotide-based agents inhibiting HIF-1, mostly by specifically targeting HIF-1α synthesis and/or degradation, have been identified and developed[13,14]; among these, thioredoxin-1 (Trx-1) inhibitors, such as pleurotin, 1-methylpropyl 2-imidazolyl disulphide (PX12) and the quinol compounds PMX290 and PMX464, have been shown to decrease HIF-1α levels and/or HIF-1 activity in a number of different cancer cell lines.[15,16]

In the present study, we investigate the role of HIF-1 in the response of HCT116 human colon adenocarcinoma cells to 5-fluorouracil (5FU), a cornerstone in the polychemotherapeutic management of this tumour type, and we assess the ability of the fluorinated, indole-substituted quinol compound PMX290 to increase the cytotoxic activity of 5FU in this cell line following HIF-1 upregulation. Our results indicate that when HIF-1 activity is increased, either by exposing cells to hypoxia or by forced expression of a degradation-resistant form of HIF-1α, cell response to 5FU is significantly impaired; conversely, knockdown of HIF-1α by RNA interference partially prevents hypoxia-induced resistance to 5FU. Similarly, when HCT116 cells are grown as three-dimensional spheroids, a model which more closely reproduces the hypoxic conditions occurring in solid tumours, the response to 5FU is significantly impaired. PMX290 inhibits HIF-1 activity in HCT116 cells, grown both as hypoxic monolayers and as spheroids, and concomitantly potentiates the effect of 5FU in both models. These observations suggest that quinol compounds, by targeting cells that present increased HIF-1activity, might help improve the success rate of 5FU-containing regimens for the management of colon cancer.

2. Materials and methods

2.1. Chemicals and reagents

5FU, standard chemicals and cell culture reagents were purchased from Sigma–Aldrich s.r.l. (Milan, Italy), unless otherwise indicated. PMX290 (formerly AJM290) was kindly provided by Prof. M.F. Stevens, University of Nottingham and Pharminox Ltd., UK.

2.2. Cell cultures, induction of hypoxia and drug treatment

HCT116 and H630 human colon cancer cells were obtained from American Type Culture Collection (Rockville, MD, USA). Both cell lines have been extensively characterised. Importantly, they are known to differ in at least two features that might affect their response to 5FU: (a) HCT116 cells harbour a wildtype *p53* gene,[17] whereas in H630 a mutant form of the gene is present,[18] which might impair the response of the latter cell lines to fluoropyrimidines[19]; (b) HCT116 cells have a homozygous wildtype genotype (2R/2R) as regards the number of tandem repeats of a 28 bp sequence in the enhancer region at the 5′UTR of the thymidylate synthase (TS) gene, encoding the major target for 5FU action,[17] whereas H630 cells have a 3R/3R genotype,[18] a common polymorphism that is associated with increased TS activity and decreased response to 5FU.[20] Another polymorphism has been reported in HCT116 cells, namely, a homozygous 6bp deletion in the 3′UTR of the TS gene (–6 bp/1494),[17] which has been associated with reduced stability and translation of the TS mRNA (another feature that generally results in improved cell response to 5FU).[21] In addition, HCT116 cells have been shown to express very low levels of dihydropyrimidine dehydrogenase (the major 5FU-inactivating enzyme), whereas thymidine phosphorylase (one of the enzymes concurring to the anabolic activation of 5FU) is clearly detectable.[22] HCT116/HRP-EGFP cells were obtained from HCT116 cells by transfection 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 HRE of the human VEGF gene and a human cytomegalovirus (CMV) minimal promoter (kindly provided by Dr. Y. Cao).[23,24] Stably transfected clones were selected after two weeks of growth in media containing 500 μg/ml of the G418 antibiotic; the clone with the highest EGFP induction was used for the experiments. The 293FT cell line (Invitrogen, Milan, Italy), was used to produce replication-incompetent lentiviral particles. In order to obtain three-dimensional spheroids, HCT116 and HCT116/HRP-EGFP cells, grown as monolayers, were detached by trypsinisation, seeded (5×10^3 cells/well) onto 96-well tissue culture plates coated with 1.5% agarose in complete medium and incubated at 37 °C in a 5% CO_2 atmosphere. Spheroids were collected at 7 d of growth and used for flow cytometric and cytotoxicity studies.

All cell lines were maintained in DMEM supplemented with 10% foetal bovine serum (EuroClone, Italy), 1% glutamine, 1% antibiotic mixture, 1% sodium pyruvate and 1% non-essential aminoacids at 37 °C in a humidified 5% CO_2 atmosphere. For all experiments, cells were exposed to 5FU and/or PMX290 for 48 h; where appropriate, hypoxia was induced during the last 24 h by placing the cells into a modular incubator chamber (Billups Rothenberg Inc., Del Mar, CA, USA) flushed with a mixture of 1% O_2, 5% CO_2 and 94% N_2 at 37 °C.

2.3. Construction of lentiviral vectors

Lentiviral particles were generated using a second-generation transient expression system, composed of (i) the pCMVΔR8.74 packaging construct, (ii) the pMD2.G envelope expression construct and (iii) task-specific lentiviral vectors: (a) the pWPT/GFP transfer vector, for overexpression of a HIF-1α degradation-resistant mutant cDNA or (b) the pLVTHM/GFP transfer vector, for silencing of HIF-1α expression by RNA interference. The plasmids pWPT/GFP and pLVTHM/GFP

contain a green fluorescent protein (GFP) cDNA under the transcriptional control of an intronless human elongation factor 1-α (EF1-α-short) promoter. All constructs were kindly provided by Dr. Didier Trono (School of Life Sciences, Swiss Institute of Technology, Lausanne, Switzerland; http://trono-lab.epfl.ch/). The transfer vector pWPT/HIF-1αMUT/GFP was generated by cloning a 3100-bp fragment containing the cDNA of a human mutated form of HIF-1α (kindly provided by Dr. Chris Paraskeva, University of Bristol, UK) into the pWPT/GFP vector. In HIF-1αMUT, Pro402 and Pro564 have been replaced by alanine and glycine, respectively; these modifications prevent oxygen-dependent prolyl hydroxylation, so that the protein is degradation-resistant under normoxic conditions.[25] The transfer vector pLVTHM/shHIF-1α/GFP was generated as follows: a sense strand of 19 nucleotides specific for HIF-1α, preceded by overhangs specific for *MluI* cloning, was designed to be followed by a short loop sequence (ttcaagaga), by the reverse complement of the sense strand, with five final thymidines to act as a RNA polymerase III transcriptional stop signal, and by a sticky sequence specific for *ClaI*. The forward oligonucleotide (5′-gcgtccccACgTTgTgAgTggTATTATTtcaagagaATAATACCACTCACAACgTtttttggaaat-3′), starting at nucleotide 1017 of HIF-1α, was annealed with a complementary reverse oligonucleotide (3′-aggggTGCAACACTCACCATAATAaagttctct-TATTATGGTGAGTGTTGCaaaaaccttagc-5′[26] in annealing buffer (100 mM potassium acetate, 30 mM HEPES, pH 7.4, 2 mM magnesium acetate). Annealed oligonucleotides were phosphorylated with PNK kinase (Fermentas, Italy) and cloned into the *MluI-ClaI* sites of a pLVTHM lentiviral vector.

2.4. Generation of lentiviral particles and target cell infection

Lentiviral particles pseudotyped with the VSV envelope glycoprotein were produced by co-transfecting 5×10^6 293FT cells with 40 μg of total plasmid DNA: the (i) pCMVΔR8.74, (ii) pMD2.G and (iii) pWPT/HIF-1α MUT/GFP or pLVTHM/shHIF-1α/GFP vectors, with the calcium phosphate precipitation method, as previously described.[27] Transduction experiments were performed in a medium containing 4 μg/ml polybrene. Viral titration was performed by flow cytometer-counting GFP-expressing HCT116 cells 48 h after infection. For *in vitro* mutant-HIF-1α overexpression experiments and shRNA-HIF-1α silencing, 30% confluent HCT116 cells were infected for 4 h with 10 MOI lentiviral particles; the particle-containing medium was then replaced with fresh medium and cells were incubated at 37 °C for 48 h before use.

2.5. Assessment of HIF-1α expression by Western blot analysis

Western blot analysis was carried out to detect the expression of HIF-1α in whole cell lysates, following normoxic or hypoxic incubation and/or drug treatment. Cells were lysed in a buffer containing NaCl 120 mM, NaF 25 mM, EDTA 5 mM, EGTA 6 mM, sodium pyrophosphate 25 mM in TBS 20 mM, pH 7.4, PMSF 2 mM, Na_3VO_4 1 mM, phenylarsine oxide 1 mM, 1% v/v NP-40 and 10% v/v Protease Inhibitor Cocktail. Protein concentration was determined by the BCA assay (Pierce, Italy) and 100 μg of protein per sample was loaded onto polyacrylamide gels (8%) and separated under denaturing conditions. Protein bands were then transferred onto Hybond-P membranes (Amersham Biosciences, Italy) and Western blot analysis was performed by standard techniques with mouse monoclonal anti-human HIF-1α antibody (BD Biosciences; dilution, 1:300). Equal loading of the samples was verified by re-probing the blots with a mouse monoclonal anti-actin antibody (Santa Cruz Biotechnology Inc.; dilution, 1:1000). Protein bands were visualised using a peroxidase-conjugated anti-mouse secondary antibody (Sigma–Aldrich; dilution, 1:4000) and the Supersignal West Femto Maximum Sensitivity Substrate (Pierce, Italy).

2.6. Effects on cell growth

The antiproliferative effects of 5FU were assessed based on cell counts. Cells (3.5×10^4/well) were seeded onto 24-well plates, allowed to attach and grow for 24 h and subsequently exposed to different 5FU concentrations (0.5–250 μM for HCT116, H630, HCT116/pLV and HCT116/shRNA-HIF-1α cells; 25–500 μM for HCT116/WPT and HCT116/HIF-1α MUT cells). After 48 h incubation in the presence of 5FU under normoxic or hypoxic conditions (for the last 24 h), cells were harvested and counted using a Beckman Coulter Z series cell counter (Beckman Coulter, Fullerton, CA, USA). To determine the sensitivity of spheroids to 5FU, the clonogenic assay was used. Following 48 h treatment with 5FU (0.5 and 1 mM) and/or PMX290 (2.5 μM), spheroids were disaggregated using a Trypsin-EDTA solution, counted and 150 cells/well were plated onto six well plates and allowed to grow for 8 d. At the end of this period, cell colonies were fixed with 95% v/v methanol for 15 min at room temperature, and stained with a solution of methylene blue in 80% v/v ethanol for 1 h. Only colonies consisting of more than 50 cells were scored.

IC_{50} values were calculated by the median effect equation.[28]

2.7. Flow cytometric analysis

HIF-1α activity was assessed in HCT116/HRP-EGFP cells grown as monolayer cultures (under normoxic or hypoxic conditions) or as spheroids, with or without PMX290 (0.25 and 0.5 μM for monolayer cells and 2.5 μM for spheroids) for 48 h. At the end of the treatment, cells from monolayers were harvested, resuspended in PBS and immediately analysed by flow cytometry. Spheroids were disaggregated as described above, resuspended in PBS and analysed. Induction of apoptotic cell death under different experimental conditions was also evaluated by flow cytometry. Monolayer cells were exposed for 48 h to 5FU (10–500 μM), alone or in combination with PMX290 (0.25 and 0.5 μM), under normoxic or hypoxic conditions; subsequently, cells were harvested by trypsinisation (pooling adherent and detached cells), washed in PBS and fixed in 70% v/v ethanol at –20 °C. Spheroids were incubated for 48 h in the presence of 5FU (0.5–1.0 mM), with or without PMX290 (2.5 μM), disaggregated by trypsinisation and processed as described for monolayer cells. After a further wash with PBS, DNA was stained with 50 μg/ml propidium iodide (PI) in PBS in the presence of RNAse A (30 U/ml) at 37 °C for 30 min. All the samples were analysed with a

FACScan flow cytometer (Becton Dickinson Mountain View, CA, USA), equipped with a 15 mW, 488 nm and an air-cooled argon ion laser. At least 10,000 events were analysed for each sample and all data were processed using CellQuest software (Becton Dickinson). EGFP (enhanced green fluorescent protein) fluorescence data were collected, using log amplification, in the FL1 channel (530/30) and fluorescence intensity was expressed as mean fluorescence channel (MFC). Fluorescent emission of PI was collected through a 575 nm band-pass filter acquired in *log* mode and the percentage of apoptotic cells in each sample was determined based on the sub-G1 peaks detected in monoparametric histograms.

2.8. Statistical analysis

Student's *t* test was used to evaluate the difference between 5FU IC_{50} values under normoxic versus hypoxic conditions in monolayer cultures and in monolayers versus 3D cultures. All the other data were analysed by two-way analysis of variance with Bonferroni post-test for multiple comparisons, using Prism 4.03 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Effect of hypoxia on human colon carcinoma cell response to 5FU

Hypoxic incubation of HCT116 cells, grown as monolayers, during the last 24 h of a 48-h treatment with 5FU significantly inhibits both the antiproliferative and proapoptotic effects of the fluoropyrimidine. The IC_{50} value calculated for hypoxic HCT116 cells was 13.17 ± 2.57 μM, whereas in normoxic cells a value of 7.15 ± 2.69 μM was obtained (means ± SE of four independent experiments; $p < 0.05$). Similar results were obtained in another human colon carcinoma cell line, H630, under identical conditions, with IC_{50} values of 10.77 ± 1.69 μM under normoxic conditions and 20.28 ± 3.6 μM in hypoxic cells (means ± SE of four independent experiments; $p < 0.05$); interestingly, in spite of the reported differences in p53 and TS activity,[17,18] the two cell lines do not significantly differ as regards their response to 5FU under normoxic and hypoxic conditions. Fig. 1A shows the percentage of apoptotic HCT116 cells following 48 hours exposure to 5FU (10, 100 and 250 μM) under normoxic and hypoxic conditions (during the last 24 h): again, hypoxic cells are less responsive than their normoxic counterparts, showing a significant reduction in apoptotic cells. Western blot analysis (Fig. 1B) confirms that following 24 h exposure to 1% pO_2, HIF-1α protein levels increase as compared to normoxic HCT116 cells, where HIF-1α protein is barely detectable; HIF-1 transcriptional activity is also significantly increased following hypoxic exposure, as indicated by the rightwards shift of the fluorescence peak obtained by flow cytometry in HCT116/HRP-EGFP, in which EGFP expression is controlled by a hypoxia-responsive promoter (Fig. 1C).

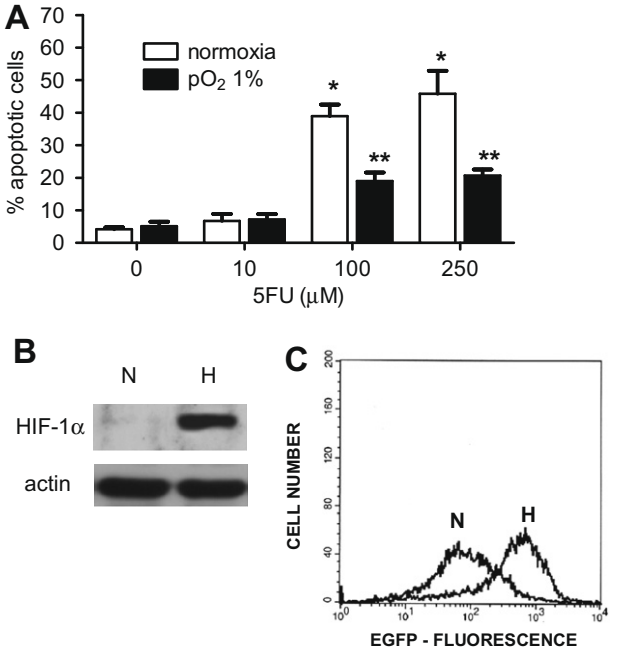

Fig. 1 – Effect of hypoxia (24 h) in HCT116 cells on (A) 5-fluorouracil (5FU)-induced apoptosis (mean ± SE, $n = 3$; $^{*}p < 0.001$ versus normoxic controls; $^{**}p < 0.01$ versus hypoxic controls and $p < 0.001$ versus normoxic cells at the same 5FU concentration); (B) HIF-1α protein levels; (C) HIF-1 transcriptional activity (see text).

3.2. HIF-1α protein levels modulate the response to 5FU

To assess the role played by HIF-1 in the observed diminished response of hypoxic HCT116 cells to 5FU, we engineered two different cell lines by infecting HCT116 cells with lentiviral vectors. HCT116/HIF-1α MUT cells express a mutated, degradation-resistant form of HIF-1α, which results in increased protein levels under normoxic condition, as compared to HCT116 cells transduced with the control vector WPT (Fig. 2A). IC_{50} values obtained following 48 h exposure to 5FU under normoxic conditions are 658.58 ± 62.6 μM in HCT116/HIF-1α MUT cells versus 6.55 ± 1.46 μM in HCT116/WPT cells (mean ± SE of 3 independent experiments, $p < 0.001$). The proapoptotic effect of 5FU is also impaired in HCT116/HIF-1αMUT cells, whereby even very high drug concentrations (250–500 μM) do not induce a significant increase in apoptotic cells, whereas a significant and dose-dependent increase is observed in HCT116 cells infected with the control vector (Fig. 2B). In HCT116/shRNA-HIF-1α cells, induction of HIF-1α protein expression following hypoxic incubation is inhibited, although not completely blocked, by RNA interference (Fig. 3A). The following IC_{50} values were estimated from the dose-response curves obtained following 48 h exposure to 5FU: HCT116/pLV cells (infected with the control vector), 10.93 ± 4.18 μM in normoxia versus 26.49 ± 5.87 μM in hypoxia (mean ± SE of three independent experiments, $p < 0.05$); HCT116/shRNA-HIF-1α, 8.21 ± 3.48 μM in normoxia versus 5.83 ± 1.58 μM in hypoxia (non-significant). These values indicate that knockdown of HIF-1α prevents the development of hypoxia-induced resistance to 5FU. Accordingly, apoptotic cell death induced by 5FU under hypoxic conditions is increased by knockdown of HIF-1α levels, whereas this modification does not affect the response of normoxic cells (Fig. 3, panels B and C).

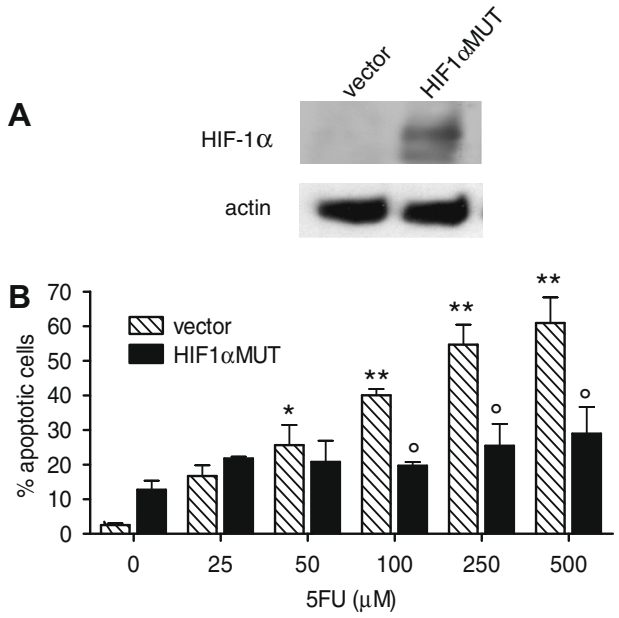

Fig. 2 – Effect of HCT116 cell transduction with the HIF-1αMUT or WPT (control) lentiviral vectors[25] under normoxic conditions on (A) HIF-1α protein levels; (B) 5FU-induced apoptosis (mean ± SE, $n = 3$; $^{*}p < 0.05$ versus untreated HCT116/WPT cells; $^{**}p < 0.001$ versus untreated HCT116/WPT cells; and $^{\circ}p < 0.01$ versus HCT116/WPT cells at the same 5FU concentration).

3.3. *Effects of thioredoxin-1 inhibitor PMX290 on HIF-1 activity and tumour cell response to 5FU*

Fig. 4A shows representative tracings obtained by flow cytometric analysis of the fluorescent emissions due to HIF-1-dependent expression of EGFP in HCT116/HRP-EGFP cells, following 48 h exposure to 0.25 μM of the Trx-1 inhibitor PMX290, in normoxic and hypoxic conditions, expressed as mean of EGFP fluorescence (the same analysis was performed on HCT116/HRP-EGFP cells treated with 0.5 μM PMX290, but as the tracing was largely superimposed to that obtained at 0.25 μM, it was omitted from the figure for the sake of clarity). Hypoxia induces a significant increase in HIF-1 activity, as shown by the rightwards shift of the corresponding fluorescent peak; following treatment with both PMX290 concentrations under hypoxic conditions, such shift is significantly inhibited, indicating a decrease in HIF-1 transcriptional activity. In contrast, HIF-1α protein levels are unaffected by PMX290 treatment, irrespective of the concentration used and of pO_2 levels (Fig. 4B). Panels C and D show the percentage of apoptotic cells, obtained by flow cytometric analysis of HCT116 cells exposed to 5FU and PMX290 under normoxic (C) and hypoxic (D) conditions. The results of these experiments indicate that treatment with PMX290 alone increases the percentage of apoptotic cells in a concentration-dependent fashion, more markedly under hypoxic than under normoxic conditions. When PMX290 is combined with 5FU under normoxic conditions, no significant differences from treatment with 5FU alone can be observed; however, combined treatment partially restores the proapoptotic effect of 5FU when this is reduced following hypoxic incubation.

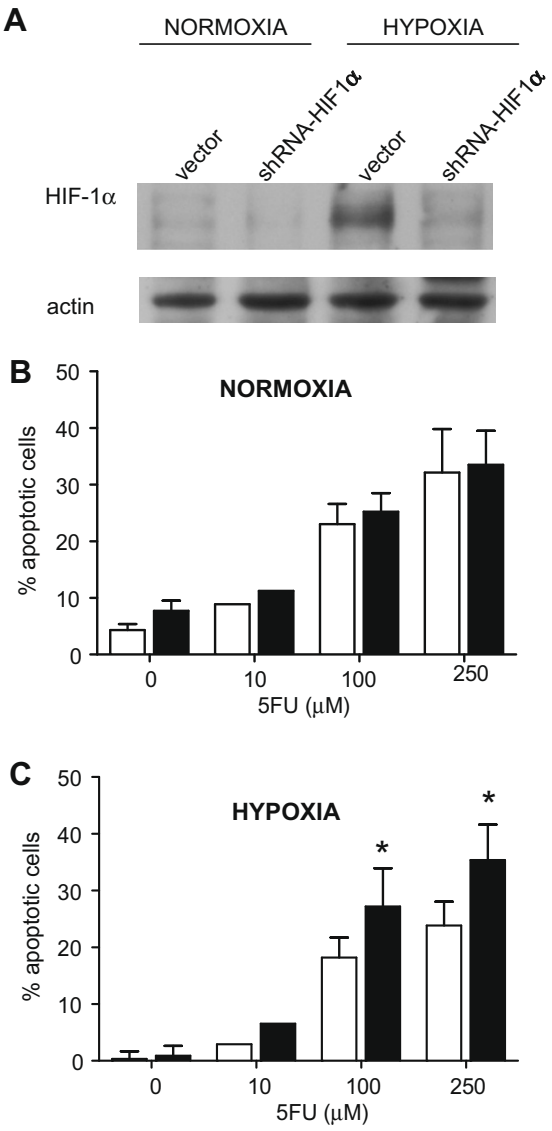

Fig. 3 – Effect of HCT116 cell transduction with the shRNA-HIF-1α or pLV (control) lentiviral vectors on (A) HIF-1α protein levels under normoxic or hypoxic conditions; (B) and (C) 5FU-induced apoptosis under normoxic (B) or hypoxic (C) conditions (see text for details). Empty bars: control vector; filled bars: shRNA-HIF-1α vector (mean ± SE of three independent experiments. $^{*}p < 0.05$ versus HCT116/pLV cells at the same 5FU concentration).

3.4. *Effect of 5FU and PMX290 on HCT116 cells grown as 3D-spheroids*

Three-dimensional cultures were obtained from HCT116 and HCT116 HRP/EGFP cells. Following 7 d incubation in non-adherent conditions, spheroids are formed, with diameters ranging from 500 to 600 μm (not shown). Flow cytometric analysis of cells from HCT116 HRP/EGFP spheroids shows that fluorescence intensity is significantly higher in these cells than in monolayer cells (Fig. 5A), indicating higher HIF-activity. Accordingly, 5FU is significantly less potent in inhibiting the clonogenic potential of cells derived from HCT116 spheroids than it is when tested on cells from monolayer cultures,

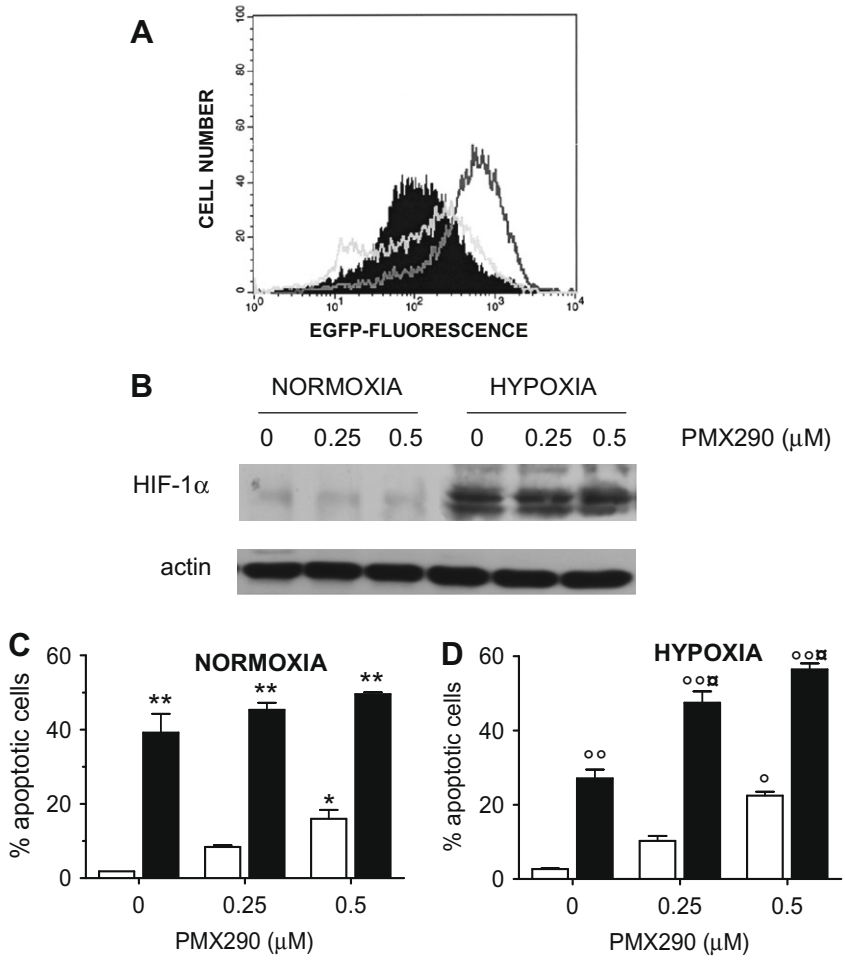

Fig. 4 – Effect of PMX290 on (A) HIF-1 transcriptional activity in HCT116/HRP cells. Shaded peak: normoxic control (untreated); dark grey outline: hypoxic control (untreated); light grey outline: PMX290 (0.25 μM for 48 h) under hypoxic conditions; (B) HIF-1α protein levels under normoxia or hypoxia condition; (C) 5FU-induced apoptosis under normoxia (*$p < 0.05$ versus untreated normoxic cells; **$p < 0.05$ versus normoxic cells without 5FU at the same PMX290 concentration) and (D) under hypoxia conditions (°$p < 0.01$ versus untreated hypoxic cells. °°$p < 0.001$ versus normoxic cells without 5FU at the same PMX290 concentration; ¤$p < 0.01$ versus hypoxic cells treated with 5FU alone. Empty bars: no 5FU; filled bars: 500 μM 5FU for 48 h). Mean ± SE, $n = 3$.

with IC_{50} values of 132.76 ± 41.04 and 12.26 ± 4.76, respectively, (means ± SE of three experiments; $p < 0.001$). Fig. 5B shows the effect of PMX290 2.5 μM for 48 h (which does not significantly reduce cell) on cell fluorescence, i.e. on HIF-1 activity. The histogram representing untreated cells exhibits a slight shoulder at low fluorescence intensity values (5A); following PMX290 incubation, two cell subpopulations become clearly visible, indicating that a significant fraction of cells has shifted towards lower fluorescence intensities. Flow cytometric analysis of apoptotic cells from spheroids exposed to 5FU with or without PMX290 (Fig. 5C) shows a significant increase in apoptosis induction by 5FU (0.5–1.0 mM for 48 h) following combined exposure with the quinol (2.5 μM) versus exposure to 5FU alone.

4. Discussion

The presence of chronic hypoxia has been demonstrated in many solid tumours, including colon carcinomas,[29] and represents a major obstacle to the success of chemotherapy. Hypoxic tumour cells are protected against apoptosis induced by a number of currently used anticancer agents, such as etoposide, vincristine and carboplatin.[6,30,31] Our data show that two different hypoxic human colon carcinoma cell lines, HCT116 and H630, are more resistant than their normoxic counterparts to the cytotoxic action of 5FU. Resistance to 5FU under hypoxic conditions had previously been reported in PC12 rat pheochromocytoma cells by Alvarez-Tejado and colleagues,[32] who, however, did not specifically investigate the role played by HIF-1 in this phenomenon. The comparison between the two cell lines examined, which have been shown to differ in some important aspects potentially involved in cell response to 5FU,[17,18] indicates that the presence or absence of a functional p53 protein, as well as different baseline levels of TS activity (due to the presence of different polymorphic alleles at the 5′UTR enhancer region of the TS gene) have no significant impact on the effect of hypoxia on drug response. The mechanism underlying decreased drug response under

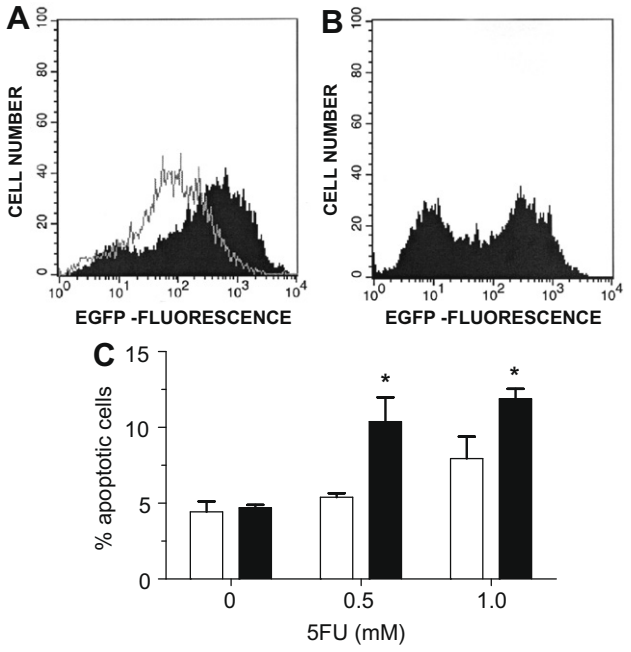

Fig. 5 – (A) HIF-1 transcriptional activity in HCT116 HRP/EGFP monolayers (grey outline) or 3D-spheroids (black peak). (B) and (C) Effect of PMX290 on HIF-1 transcriptional activity in HCT116 HRP/EGFP spheroids (B) and on 5FU-induced apoptosis in spheroid-derived cells (*$p < 0.05$ versus cells from spheroids treated with 5FU alone. Empty bars: no PMX290; filled bars: 2.5 μM PMX290 for 48 h).

hypoxia is very likely multifactorial, involving both HIF-1-dependent and -independent effects. In our experimental model, HIF-1 activation clearly makes a major contribution to the resistance of hypoxic HCT116 cells to 5FU, as shown by different orders of evidence. First, incubation under hypoxic conditions inducing resistance to 5FU leads to accumulation of HIF-1α protein and to an increase in HIF-1 transcriptional activity, as assessed in HCT116 cells stably transfected with a reporter plasmid expressing EGFP under the control of multiple copies of the HRE derived from the human VEGF gene promoter. Second, HIF-1α knockdown by infection of HCT116 cells with a lentiviral vector encoding a shRNA that specifically mediates degradation of HIF-1α mRNA partially prevents the development of resistance to 5FU under hypoxic conditions. Finally, infection of HCT116 cells with a lentiviral vector encoding a mutant form of HIF-1α, lacking the two proline residues that are critical for HIF-1α oxygen-dependent hydroxylation and subsequent degradation, leads to resistance to 5FU under normoxic conditions.

Why are hypoxic HCT116 cells less responsive than normoxic cells to 5FU? A number of authors have shown increased expression of both ABCB1 (also known as P-glycoprotein) and/or ABCG2 (also known as BRCP) drug efflux transporters in hypoxic cells,[7,8] which may account for lower efficacy of a broad range of anticancer agents; however, 5FU is not a known substrate for either transporter, and therefore this mechanism is not likely to play a major role in 5FU resistance. Impairment of mismatch repair activity could be involved in hypoxia-induced resistance to a number of agents requiring activation of this DNA repair system to effectively generate a death signal, including 5FU.[33,34] However, HCT116 cells are known to be intrinsically defective in mismatch repair, due to a homozygous mutation in the mismatch repair gene hMLH1 on chromosome 3[35]; thus, modulation of this or other components of the mismatch repair system, which has been described by Koshiji and colleagues,[9] should be irrelevant to the response of this cell line. Finally, it is well known that increases in the expression/activity of TS and dihydropyrimidine dehydrogenase, as well as a decrease in thymidine phosphorylase may negatively affect tumour cell response to 5FU *in vitro*.[36,37] This issue has not been specifically addressed in the present study; however, a number of reports in the literature seem to indicate that hypoxia tends to decrease TS and DPD expression,[38] and to increase TP expression,[39] which overall should increase, rather than decrease, cell sensitivity to 5FU. Therefore these reports suggest that if any such modulation of 5FU-related enzymes occurs under hypoxic conditions, it is superseded by other, more potent effects impairing drug response. Thus, the most likely hypothesis to explain the reduced potency of 5FU in hypoxic HCT116 cells involves critical alterations in the levels of pro- and antiapoptotic factors. An increasing body of experimental evidence indicates that HIF-1 activation modulates the expression of pro- and antiapoptotic genes; significantly increased levels of survivin, IAP-2, Bcl2, Bcl-x_L and Mcl-1, as well as decreased levels of Bax and Bad, have been reported in tumour cells derived from liver, breast, pancreas and squamous cell cancers.[11,40–43] Regarding colon cancer cells, Erler and colleagues have reported that in HCT116 cells, HIF-1 exerts its antiapoptotic effect by downregulating the proapoptotic protein Bid.[12] More recently, Kulshreshtha and colleagues have demonstrated that besides directly regulating the expression of pro- and antiapoptotic genes, in HCT116 cells HIF-1 also affect the expression of a vast array of microRNAs; more specifically, a number of miRNAs that negatively regulate proapoptotic signals have been found to be induced under hypoxic conditions via a HIF-1-dependent mechanism.[10,44] An imbalance between pro- and antiapoptotic signals favouring cell survival could very well account for a reduced response to a wide range of anticancer agents and thus could explain the resistance to 5FU observed in hypoxic HCT116 cells; investigations are underway, aiming at identifying the role played by specific pro- and antiapoptotic factors in this experimental model.

Having established that HIF-1 is directly involved in determining hypoxia-induced resistance to 5FU in HCT116 cells, we then assessed the ability of PMX290, a fluorinated indole-substituted quinol Trx-1 inhibitor, to restore the response of HCT116 cells to 5FU. Trx-1 is a ubiquitous 12-kDa protein and the major intracellular disulphide reductase, that is overexpressed in a variety of cancer cell lines and tumours,[45] and plays a key role in the regulation of biological functions such as cell proliferation, growth control and apoptosis by modulating the actions of crucial cellular enzymes and transcription factors, including NF-κB and p53.[46] An early report suggested that Trx-1-enhances the transcriptional activity of HIF-1 by reducing critical sulphhydryl groups in its DNA binding domain.[47] However, more recent

studies indicate that the mechanism underlying HIF-1 modulation by Trx-1 is more complex than direct activation. Trx-1 has been proposed to increase HIF-1α levels, possibly by increasing its mRNA levels[48] or by inhibiting its degradation[49] or by a combination of both mechanisms. In addition, Trx-1 has been suggested to interfere with HIF-1 interactions with transcriptional coactivators such as SRC-1/p160 and CBP/p300,[50] an effect that appears to depend on Trx-1-mediated reduction of the nuclear redox regulator Ref-1.[51] Thus, targeting Trx-1 seems a reasonable approach to decreasing HIF-1α expression and/or HIF-1 activity. Accordingly, a number of small molecule inhibitors have been synthesised, which are reported to decrease both Trx-1 and HIF-1 levels and/or activity. More specifically, the quinol compounds PMX290 (formerly known as AJM290) and PMX464 (formerly known as AW464) have been reported to significantly inhibit Trx-1 by irreversibly binding the sulphhydryl groups of two crucial cysteine residues in its catalytic motif (Cys32 and Cys35)[52]; this is accompanied by a significant decrease in HIF-1 transcriptional activity in breast carcinoma cells (whereas HIF-1α levels were found to be paradoxically increased by the same compounds).[16] Our results indicate that PMX290 also decreases HIF-1 activity in colon-derived carcinoma cells grown as monolayers under hypoxic conditions or as three-dimensional spheroid at normoxic pO_2 values; however, HIF-1α protein levels are unaffected by the treatment, suggesting that the increase observed by other authors in breast cancer cells is possibly a cell-specific effect. Interestingly, combining 5FU with PMX290 (at concentrations that inhibit HIF-1 activity while only exerting very mild intrinsic growth inhibitory effects) significantly enhances the antiproliferative effect of 5FU, as assessed by cytotoxicity assays and significantly increases the percentage of apoptotic cells as compared with 5FU alone. Our studies on three-dimensional cultures basically confirm our findings in monolayer cells. Flow cytometric analysis of spheroids obtained from HCT116 HRP/EGFP cells, with diameters of 500–600 μm (not shown), indicates that hypoxia develops spontaneously within the cell mass, leading to HIF-1 activation (as indicated by an increase in cell fluorescence); this is accompanied by a significant decrease in the antiproliferative effect of 5FU, as assessed by the clonogenic assay on disaggregated spheroids, in comparison with monolayer cells. This effect might depend, at least in part, on restricted access of the drug to the innermost cells in the spheroids. However, the observation that subtoxic concentrations of PMX290 simultaneously reduce HIF-1 activity and enhance the response to 5FU, together with the data obtained on monolayer cells, suggests HIF-1 inhibition as a likely mechanism involved in cell sensitisation.

To conclude, our data indicate that adding PMX290 to 5FU-based chemotherapeutic regimens might present with some therapeutic benefit, due to the ability of this compound to sensitise cells within hypoxic areas of the tumour to the effects of the fluoropyrimidine.

Conflict of interest statement

None declared.

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