

Microtubule-targeting-compound PBOX-15 radiosensitizes cancer cells in vitro

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Abbreviations: PBOX-15, pyrrolo-1,5-benzoxazepines-15; HIF1 α , hypoxia-inducible-factor 1alpha

Background: We proposed to investigate the radiosensitizing properties of PBOX-15, a novel microtubule-disrupting agent, in a panel of cancer cell lines.

Results: PBOX-15 treatment was associated with significant cell kill and increased radiosensitivity in all three cell lines tested. The number of surviving cells in response to the combined treatment was significantly less than PBOX-15 alone in 22Rv1 cells. In these cells, radiosensitisation correlated with induction of G₂/M cell cycle arrest by PBOX-15. The compound sustained its activity and increased HIF-1 α expression under hypoxic conditions. PBOX-15 prevented onset of hypoxia-induced radioresistance in hypoxic prostate cells and reduced the surviving fraction of irradiated hypoxic cells to levels similar to those achieved under aerobic conditions.

Methods: Clonogenic assays were used to determine sensitivity of a panel of cancer cell lines (22Rv1, A549, U87) to PBOX-15 alone or in combination with a single 2 Gy dose fraction. Induction of cell cycle arrest and apoptosis was investigated in 22Rv1 prostate cancer cells. The cytotoxic properties of the compound under hypoxic conditions were correlated with Hypoxia Inducible Factor 1alpha (HIF-1 α) gene and protein expression levels and its radiosensitisation potential was investigated in hypoxic 22Rv1 using clonogenic assays.

Conclusions: This preliminary data identifies the potential of PBOX-15 as a novel radiosensitising agent for the management of solid tumors and eradication of hypoxic cells.

Background

Microtubule-targeting agents, such as taxanes (paclitaxel and docetaxel) and vinca alkaloids (vincristine and vinblastine), act by suppressing microtubule dynamics and disrupting mitotic spindle configuration.¹ These compounds are cell cycle specific, arresting the cell cycle in the G₂/M phase.² Because G₂/M cells are most radiosensitive, these agents have been investigated as radiosensitizers. The combination of chemotherapy and radiation therapy indeed presents a number of advantages: because radiation is a local therapy and chemotherapy targets systemic spread, spatial co-operation of both modalities may participate in reduced local failure rates, eradication of micro metastases, improved distant control, organ function preservation and decreased tumor mass prior to surgery making complete resection possible.³ A clinical limitation to these combination therapies is the acquired or intrinsic resistance of tumors to established microtubule-targeting agents.⁴

Pyrrolo-1,5-benzoxazepines (PBOX) are a family of novel compounds with anticancer properties. We have previously demonstrated that several PBOX compounds induce apoptosis in a number

cancer cell lines including those derived from leukemia cells,^{5,6} breast cancer cells^{7,8} and ovarian cancer cells.⁹ Two of the more active PBOX compounds, PBOX-6 and PBOX-15, cause depolymerisation of the microtubule network and disassembly of tubulin in vitro.⁸ We have shown that PBOX-6 inhibits tumor growth in a murine breast cancer model¹⁰ and chronic myeloid leukemia (CML) model.¹¹ Recently, we have described the apoptosis-inducing activity of PBOX-15 in ex vivo chronic lymphocytic leukemia (CLL) cells harbouring poor prognostic markers,¹² and in ex vivo chronic myeloid leukemia (CML) patient samples including those resistant to the tyrosine kinase inhibitor, STI571.¹³ Finally, with its ability to target multi-drug resistance cancer cells, PBOX-15 may represent an alternative to clinically approved compounds.¹⁴

Hypoxia is a feature of all solid tumors, and is associated with reduced sensitivity of tumor cells to both radiation and many conventional anticancer drugs, including microtubule-disrupting agents.¹⁵ In human carcinoma cell lines, hypoxia severely affected the cytotoxicity of paclitaxel, whereas docetaxel preserved its tumor cell-killing activity even at lowest concentrations.^{16, 17} Resistance has been attributed to the lack of oxygen itself¹⁵ but

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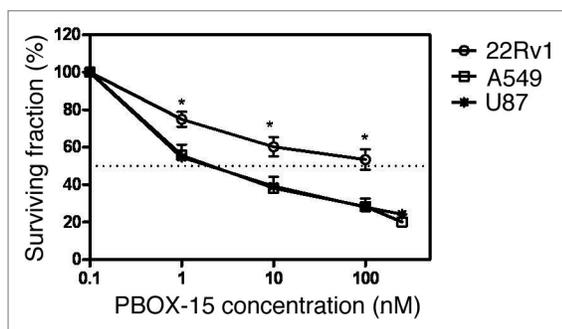


Figure 1. Prostate cancer cells are most chemoresistant to PBOX-15. Clonogenic survival of prostate (22Rv1), lung (A549) and glioma (U87) cancer cell lines to increasing concentrations of PBOX-15. N = 3; Mean \pm SEM; *p < 0.05.

also to cell cycle disturbances, inhibition of DNA double strand break repair processes and the induction of specific signaling pathways in response to hypoxic exposure.¹⁸ Induction of these pathway is regulated by the hypoxia-inducible factor 1 (HIF-1) complex. This proangiogenic transcription factor consists of a HIF-1 β and a HIF-1 α subunit, whose expression is stabilized in the absence of oxygen.¹⁹ A number of studies have reported a relationship between HIF-1 α upregulation and the response of cells to chemotherapeutic agents and ionizing radiation. HIF-1 α was recently proposed as a marker for radiosensitivity.²⁰ However, conflicting results suggest HIF-1 α can be associated with both increased and decreased radiation resistance.²⁰

HIF-1 α protein levels are elevated in normoxic cells undergoing physiological processes involving large-scale microtubule reorganization, such as embryonic development, wound healing and tumor cell metastasis.²¹ Because many chemotherapeutic drugs disrupt the microtubular system, it may be proposed that HIF-1 α overexpression in hypoxic cells may reduce the effectiveness of these compounds. A number of studies have reported involvement of HIF-1 α dysregulation in the cytotoxicity of chemotherapeutic drugs disrupting the microtubular system, such as docetaxel, paclitaxel and vincristine. Upregulation of HIF-1 α was associated with resistance to vincristine in gastric cancer cells²² and paclitaxel in lung cancer cell lines.²³ HIF-1 α protein levels and activity were downregulated by a panel of microtubule-disrupting agents in a dose-dependent manner in human ovarian cell lines.²⁴

The mode of action displayed by PBOX-15 and its effect on microtubules emphasizes its similarity to established anti-mitotic drugs such as paclitaxel, docetaxel, vincristine and vinblastine. These drugs are also established radiosensitizers in certain cancer types indicating the potential of PBOX-15 as a novel radiosensitizer. In this study, we evaluate the cytotoxicity and radiosensitizing properties of PBOX-15 in a panel of cancer cell lines (prostate, glioma and lung).

Results

Cytotoxicity of PBOX-15 under aerobic conditions. The cytotoxicity of PBOX-15 (24 h treatment) was initially determined in a panel of cancer cell lines (22Rv1, prostate; A549, lung and U87,

Glioma) using clonogenic assays (Fig. 1). PBOX-15 significantly reduced the surviving fraction of the cell lines at all concentrations tested (unpaired Student's t-test, p < 0.05). 22Rv1 was the most resistant cell line, with an IC₅₀ of 100 nM, compared to 1 nM in both other cell lines. At 1 nM, 22Rv1 were 1.3-times more resistant to PBOX-15 than both A549 and U87 cells (ANOVA, p = 0.023).

Radiosensitizing properties of PBOX-15 in aerobic cancer cells. The radiosensitizing properties of PBOX-15 were next determined using clonogenic assays. 22Rv1, A549 and U87 cells were pre-treated with increasing concentrations of PBOX-15 for 24 hrs prior to the delivery of a single 2 Gy dose fraction (Fig. 2). The surviving fraction at 2 Gy (SF2) was used as a measure for radiosensitivity. The three cell lines had similar radiosensitivities (ANOVA, p = 0.84) with SF2s of 53 \pm 2.4% (22Rv1), 51.2 \pm 3.9% (A549) and 58.2 \pm 2.6% (U87). Pre-irradiation treatment with PBOX-15 significantly reduced the SF2 of all three cell lines at both 10 and 100 nM. Clonogenic survival to PBOX-15 concentration of 250 nM and 1 μ M was not evident and resulted in no surviving fraction (data not shown). The increase in radiosensitivity at 10 nM was highest in 22Rv1 (2.3-fold), compared with A549 (0.99-fold) and U87 (1.58-fold) cells. At 10 nM, PBOX-15 reduced the SF2 of 22Rv1 from 53 \pm 2.4% to 23.6 \pm 10% (unpaired Student's t-test, p = 0.04). In these cells, the surviving fraction of cells exposed to the combined treatment was significantly less than that of cells treated with either modality alone, (ANOVA, p = 0.012 at 1 nM; p = 0.019 at 10 nM; p = 0.001 at 100 nM) (Fig. 2A). While PBOX-15 enhanced the radiosensitivity of A549 (unpaired Student's t-test, p = 0.03 at 10 nM; p = 0.002 at 100 nM) and U87 cells (unpaired Student's t-test, p = 0.01 at 10 nM; p = 0.005 at 100 nM), the surviving fraction of cells exposed to the combined treatment was not statistically different from that of cells treated with PBOX-15 alone (unpaired Student's t-test, A549: p = 0.74 at 10 nM, p = 0.82 at 100 nM; U87: p = 0.49 at 10 nM; p = 0.17 at 100 nM), (Fig. 2B and C).

Cell cycle arrest and induction of apoptosis by PBOX-15. The most resistant cancer cells to the cytotoxic effect of PBOX-15, 22Rv1 prostate cancer cells showed the largest response to the combined chemo-radiation treatment. We thus next evaluated the mechanism underlying the radiosensitizing properties of PBOX-15 in these cells. We proposed for this effect to result from induction of cell cycle arrest by PBOX-15 in the radiosensitive G₂/M phase and post-irradiation increase in cell death. To test this hypothesis, we first determined the cell cycle distribution of the cell population following exposure to PBOX-15 treatment, a 2 Gy dose fraction, or their combination (Fig. 3A). Cell cycle analysis demonstrated the induction of a G₀/G₁ cell cycle arrest at 24 hrs following irradiation (unpaired Student's t-test, p = 0.04). Treatment with 1 μ M PBOX-15 for 24 hrs induced a G₂/M phase arrest (unpaired Student's t-test, 0.25 μ M p = 1, 1 μ M p = 0.01), with an increase in the sub-G₀ population suggesting induction of apoptosis. In response to the combination treatment, the G₂/M cell population was significantly less than that of cells treated with either modality alone at all PBOX-15 concentrations tested (0.25 μ M: ANOVA, p = 0.006; 1 μ M ANOVA, p = 0.0008). This reduction correlated with a concomitant increase in G₀/G₁ phase cells (0.25 μ M: ANOVA, p = 0.002;

1 μ M ANOVA, $p = 0.04$). No effect was seen in cells treated with 10 nM or 100 nM PBOX-15 (data not shown).

Second, we assessed the induction of apoptosis by PBOX-15 in these 22Rv1 cells using caspase-3/7 activity as a marker (Fig. 3B). A significant increase in apoptosis was measured in cells treated with 1 μ M PBOX-15 for 24 hrs (unpaired Student's t -test, $p = 0.0005$) which was prevented in cells pre-treated with 50 μ M z-IETD-fmk, a caspase-8 inhibitor (unpaired Student's t -test, $p < 0.0001$). Combination with radiation did not further increase apoptosis levels in PBOX-15 treated cells (unpaired Student's t -test, $p = 0.93$). Levels of apoptosis were comparable to those measured in cells treated with 1 μ M Vincristine or 1 μ M Nocodazole for 24 hrs alone or in combination with radiation. No effect was seen in cells treated with 10 and 100 nM PBOX-15 (data not shown).

Cytotoxicity of PBOX-15 under hypoxic conditions. We next proposed to investigate whether hypoxia could affect the cytotoxic properties of PBOX-15. 22Rv1 prostate cancer cells were the most chemoresistant of the three cancer lines tested. Prostate tumors are known to be hypoxic²⁵ and prostate cancer cells are particularly sensitive to microtubule-disrupting agents.²⁶ The cytotoxic potential of PBOX-15 under hypoxic conditions was thus further investigated in prostate cancer cells. 22Rv1 cells were treated with 250 nM PBOX-15 for 24 hrs under aerobic or hypoxic conditions and cytotoxicity was assessed using clonogenic assays (Fig. 4A). PBOX-15 maintained its cytotoxic activity under hypoxic conditions and the surviving fraction of aerobic and hypoxic PBOX-15 treated 22Rv1 was not statistically different at all concentrations tested (unpaired Student's t -test, $p = 0.98$ at 1 nM, $p = 0.54$ at 10 nM and $p = 0.15$ at 100 nM).

Radiosensitizing properties of PBOX-15 under hypoxic conditions. We next investigated whether PBOX-15 could radiosensitize hypoxic prostate cancer cells. Hypoxic 22Rv1 cells were treated with PBOX-15 for 24 hrs prior to the delivery of a 2 Gy single dose fraction and determination of the surviving fraction using clonogenic assays. While hypoxia-induced radioresistance was evident in untreated cells, with an increase in SF2 from $53.82 \pm 2.493\%$ in air to 77.20 ± 1.096 in hypoxia (unpaired student t -test, $p = 0.002$), pre-treatment with PBOX-15 prevented onset of resistance and significantly reduced the hypoxic SF2 to 38.59 ± 2.598 (unpaired Student's t -test, $p = 0.01$) (Fig. 4A). The

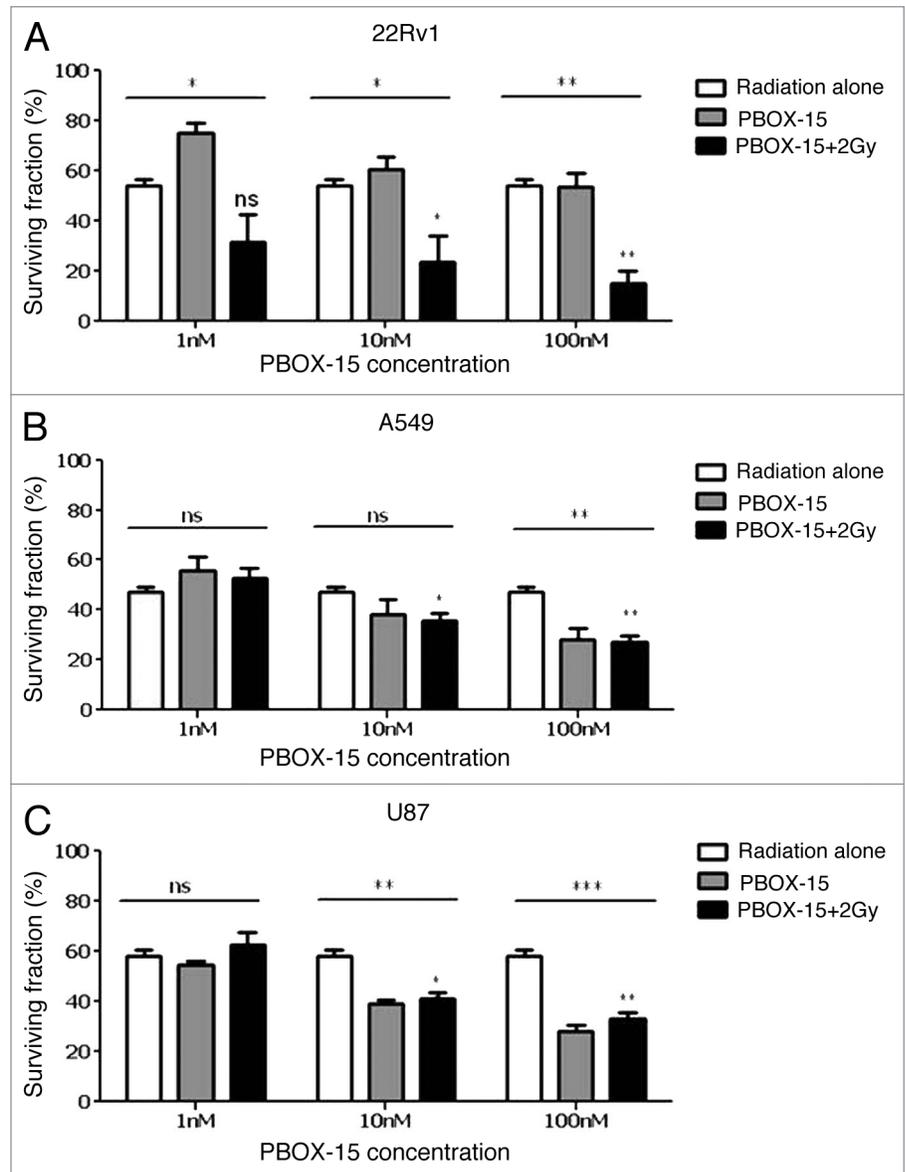


Figure 2. Prostate cancer cells are most sensitive to the radiosensitizing properties of PBOX-15. Clonogenic survival of (A) 22Rv1, (B) A549 and (C) U87 cancer cell lines pre-treated with increasing PBOX-15 concentrations prior to the delivery of a single 2 Gy dose fraction. $N = 3$; Mean \pm SEM; * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, ns = non significant.

combined effect of PBOX-15 treatment and radiation resulted in a surviving fraction that was significantly less than with either modality alone at all PBOX-15 concentrations tested (ANOVA, $p < 0.001$) (Fig. 4B).

Effect of PBOX-15 on HIF-1 α protein and mRNA levels. We finally investigated whether PBOX-15 maintained its cytotoxicity properties under hypoxic condition via deregulation of HIF-1 α expression. To test this hypothesis, we first determined HIF-1 α protein expression in untreated and PBOX-15 treated aerobic and hypoxic 22Rv1 cells (Fig. 5A). While hypoxia-selective stabilization of the HIF-1 α protein was evident, PBOX-15 (250 nM) appeared to enhance HIF-1 α protein levels under hypoxic conditions. We secondly measured relative HIF-1 α mRNA levels

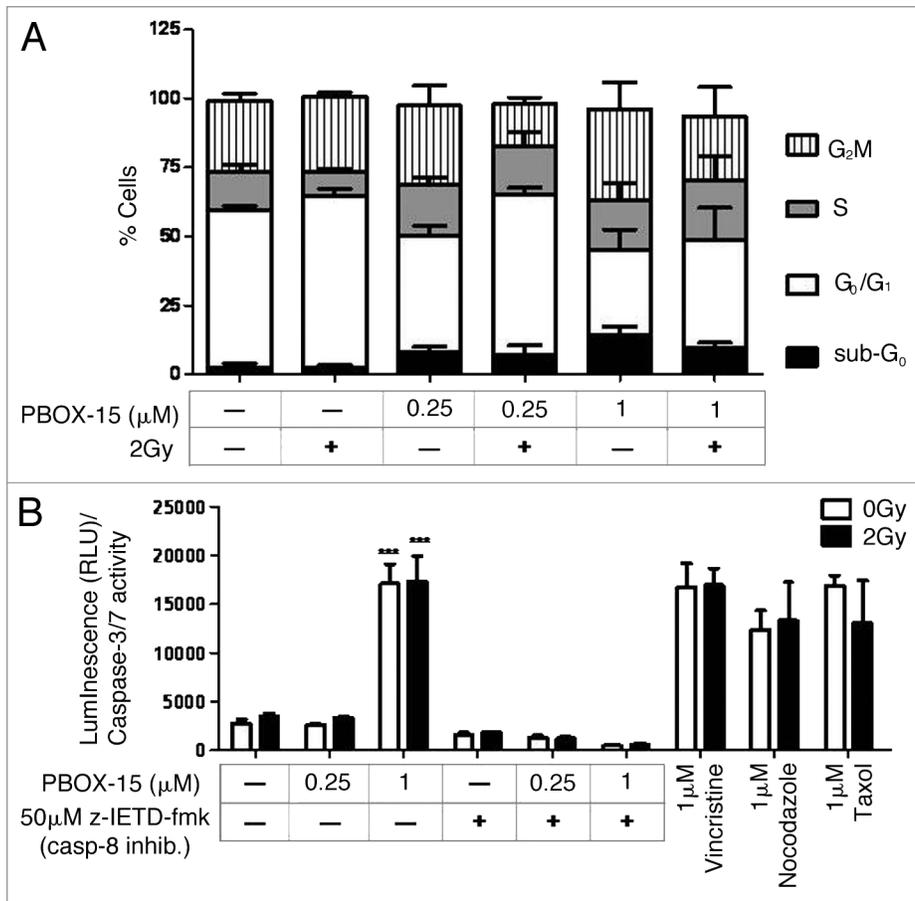


Figure 3. PBOX-15 induces G_2/M cell cycle arrest and apoptosis. (A) Cell cycle distribution analysis of 22Rv1 cells treated with PBOX-15, radiation (2 Gy) or their combination. (B) Caspase-Glo[®] 3/7 assay for the induction of apoptosis in 22Rv1 cells treated with PBOX-15, radiation (2 Gy) or their combination. N = 3; Mean \pm SEM, ***p < 0.001.

in untreated and PBOX-15 treated aerobic and hypoxic cells. HIF-1 α mRNA levels were dramatically reduced within 4 hrs of hypoxic exposure in both untreated and PBOX-15 treated cell lines (Fig. 5B). No effect was seen in cells treated with 10 nM and 100 nM (data not shown).

Discussion

Ionizing radiation participates in tumor control through the induction of mitotic cell death and inhibition of proliferation. As a result, mitotic inhibitors, such as microtubule-targeting agents, have been used as radiosensitizers. In this study, we proposed to investigate the interaction between PBOX-15, a novel microtubule depolymerising agent and radiation in the treatment of cancer cells. PBOX-15 displayed a cytotoxic effect in all three cell lines tested. The PBOX-15 concentrations (<1 μM) used in this study were previously shown to be associated with negligible bone marrow and normal tissue toxicity in a mouse model *in vivo*,¹⁰ and minimal toxicity to normal human bone marrow progenitor cells and B cells.¹² Prostate cancer 22Rv1 cells were the most chemoresistant and lung cancer A549 cells demonstrated the highest chemosensitivity of the three cell lines to PBOX-15.

The sensitivity of this panel of cell lines to PBOX-15 is similar to that observed in response to other microtubule disrupting agents, such as paclitaxel.

To investigate the potential role of PBOX-15 as a radiosensitizer, the cells were pre-treated with PBOX-15 for 24 hrs prior to irradiation. Results from these experiments demonstrated that PBOX-15 enhances the radiosensitivity of all three cell lines (Fig. 2). The cytotoxic effects of radiation and PBOX-15 were maximal in 22Rv1 cells with combined cytotoxic effect greater than that achieved with either modality alone. Enhanced radiosensitivity in 22Rv1 cells was consistent with arrest of the cells in the radiosensitive G_2/M cell cycle phase in response to PBOX-15 treatment. Subsequent exposure to radiation resulted in significant reduction in G_2/M cells and increase in the G_0/G_1 cell population. However, the lack of increase in caspase activity detected in cells treated with both PBOX-15 and radiation, compared with PBOX-15 alone, suggests that the increased cytotoxic effects observed with this treatment combination is not a result of enhanced apoptosis, but may be consistent with the induction of growth arrest or senescence.^{27, 28}

Recent evidence suggests hypoxia may act as a double-edge sword either protecting²⁹ or sensitizing¹⁵ cancer cells to therapeutic intervention. Hypoxia has long been associated with increased resistance to radiation. In this study, 22Rv1 cells were significantly more resistant to radiation under hypoxic than aerobic conditions. Hypoxia has been correlated with poor treatment outcome in prostate cancer patients.³⁰⁻³² These hypoxic regions have been detected using a number of techniques including HIF-1 α immunostaining.^{25,33,34} Recent studies have implicated HIF-1 α in radiation resistance with overexpression of HIF-1 α in oropharyngeal cancer associated with an increased risk of failure to achieve complete remission after radiation therapy.³⁵ Downregulation of HIF-1 α by microtubule-disrupting agents was recently proposed as a mechanism for increased sensitivity of hypoxic cancer cells to these agents.²³ In this study, 22Rv1 cells were as sensitive to PBOX-15 under hypoxic than aerobic conditions. This sensitivity correlated with increased HIF-1 α protein expression in hypoxic PBOX-15 treated cells. Further research is however required to characterise this correlation.

Hypoxic exposure induces a state of constant struggle between hypoxia-regulated gene expression and hypoxia-regulated translational suppression due to poor availability of ATP.³⁶ HIF-1 α mRNA levels were dramatically reduced following hypoxic exposures in both untreated and PBOX-15 treated 22Rv1 cells. The

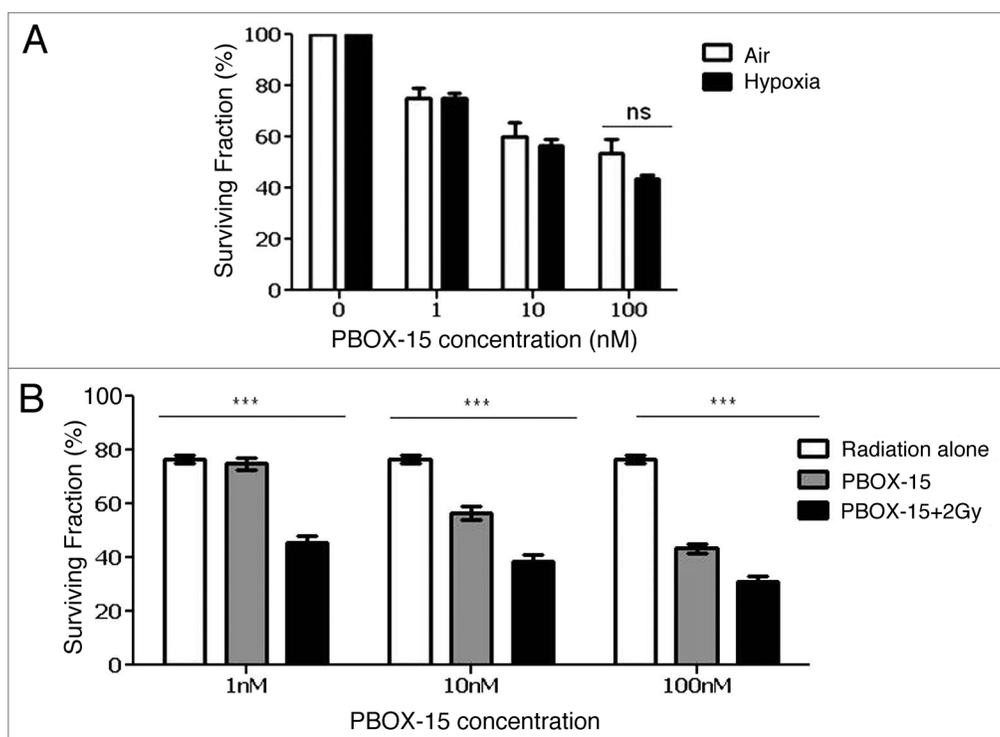


Figure 4. PBOX-15 maintains its activity under hypoxic conditions and radiosensitises hypoxic cells. (A) Clonogenic survival of 22Rv1 cells treated with increasing PBOX-15 concentrations for 24 hours under aerobic or hypoxic conditions. (B) Clonogenic survival of hypoxic 22Rv1 treated with PBOX-15, radiation (2 Gy) or their combination.

underlying mechanisms for this reduction were not investigated. While largely dependent on the inhibition of prolyl hydroxylase domain-containing proteins enzymatic activity by reduced oxygen levels,³⁷ the stabilization of HIF-1 α is regulated by a number of molecular pathways including the Harvey rat sarcomal viral oncogene homology/extra cellular signal-regulated kinase (RAS/Raf/MEK) and Protein kinase B/phosphatidylinositol-3-kinase (PI3K/AKT/PKB) pathways.³⁸⁻⁴⁰ Taxotere and MEK1/2 inhibitors were proposed to suppress mammary tumor growth in vivo. This phenomenon was enhanced by sequence-dependent exposure to ionizing radiation.⁴¹ Manipulation of the PI3K-AKT pathway by anti-HER2 antibodies trastuzumab was also proposed to potentiate the growth inhibitory activity of docetaxel by affecting cell cycle progression.⁴² The potential interaction of PBOX-15 with these pathways may have resulted in increased HIF-1 α protein levels in the present study.

The role of HIF-1 α on the therapeutic response of cancer cells is complex, with conflicting results suggesting HIF-1 α may also participate in increased therapeutic sensitivity. HIF-1 α was shown to weaken radiation-induced G₂/M arrest and force entry into mitosis, leading to a large proportion of potentially-lethal aberrant mitotic cells⁴³ and participating to enhanced radiosensitivity.⁴⁴ As a result, HIF-1 α was proposed as a marker for radiosensitivity.²⁰ Treatment with PBOX-15 prior to irradiation prevented the induction of hypoxia-induced radioresistance in 22Rv1 cells.

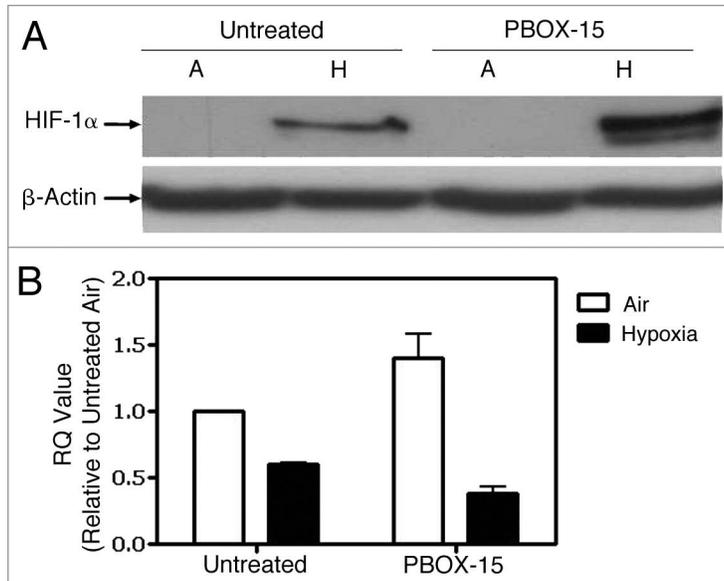


Figure 5. PBOX-15 enhances HIF-1 α protein levels. (A) Representative HIF-1 α and β -actin immunoblots of 22Rv1 cells treated with 250 nM of PBOX-15 for 6 hours under aerobic and hypoxic conditions, relative to untreated aerobic and hypoxic controls. (B) Relative quantification of HIF-1 α mRNA.

Moreover, the surviving fraction of these cells was significantly less than that either modality alone and similar to that obtained in aerobic cells. These results suggest PBOX-15 has potential for the eradication of hypoxic cancer cells during radiation therapy.

The mechanisms for this effect remain to be investigated but are likely to involve HIF-1 α dependent induction of cell cycle blockade^{45,46} and reduced clonogenicity.⁴⁷

The use of PBOX-15 as a radio-sensitizer could therefore be of benefit in patients identified as having aggressive hypoxic tumors. A number of studies have established the radio-sensitising properties of the taxane paclitaxel in head and neck cancers with some encouraging results.⁴⁸ However, significant limitations of the clinical use of taxanes exist, including acquired and intrinsic tumor resistance through the expression of multidrug resistance proteins.⁴ Other studies have recently investigated novel radio-sensitising agents in prostate cancer.⁴⁹ Here, we identify a novel microtubule targeting agent, PBOX-15, with radiosensitization activity in cancer cells. Further investigation into the effect of PBOX-15 in combination with radiation on the molecular hypoxic response may reveal if combined therapy may have therapeutic potential. As PBOX has a distinct mechanism of action compared to clinical established microtubule targeting agents and is active in multidrug resistant cancer cells,^{12,14} it warrants further investigation as a novel therapeutic agent for the radiosensitization of aerobic and hypoxic solid tumors. This compound is at a pre-clinical stage and we hope will soon be licensed to a pharmaceutical company for further development.

Materials and Methods

Cell lines. Three cell lines were used: 22Rv1 derived from a human prostatic carcinoma, A549 derived from a human non-small-cell lung carcinoma, and U-87 derived from a human malignant glioma tumor. The 22Rv1 and A549 cell lines were purchased from the ATCC biological resource centre, Middlesex, UK. The U-87 cell line was generously donated by Dr. Brian Marples, Department of Radiation Oncology, Karmanos Cancer Institute and Wayne State University, Detroit, MI. All three cell lines were cultured in RPMI 1640 media (Invitrogen, Dublin, Ireland), supplemented with 10% foetal bovine serum and 1% Penicillin/streptomycin.

Drug treatment. 4-acetoxy-5-(1-naphthyl) naphtha pyrrolo [1,4]-oxazepine (PBOX-15) was synthesized as previously described in reference 50. Paclitaxel, vincristine and nocodazole were obtained from Sigma-Aldrich, Poole, UK. All compounds were dissolved in ethanol to yield stock solutions of 1 mM and 0.1 mM and were stored at -20°C. Dilutions were made in medium before each experiment to create drug concentrations of 1, 10, 100, 250 and 500 nM.

Hypoxic and irradiation exposure. The cells were irradiated at room temperature using an Xstrahl RS225 molecular research system (Gulmay Medical Limited, Surrey, UK) at a dose rate of 3.25 Gy/minute. Hypoxia (0.5% O₂, pO₂ <2 mmHg) was achieved by exposing cells in a 1,000 in vivo hypoxic chamber (BioTrace, Bracknell, UK). The cells were exposed to a mixture of nitrogen, CO₂ (5%) and compressed air to achieve a 0.5% oxygen concentration. pO₂ was monitored with an oxygen probe (OxyLab pO₂TM, Oxford Optronix, Abingdon, UK).

Clonogenic assays. Cell survival was evaluated using a standard colony-forming assay. 1,000–10,000 cells/well were plated

onto 6-well plates prior to 24 hrs chemotherapeutic treatment under aerobic or hypoxic conditions. Two weeks later, the plates were stained (70% ethanol, Crystal violet, Sigma-Aldrich, UK) and the colonies were counted. The response of untreated cells was used as a control. The plating efficiency was calculated as the ratio of the number of colonies counted over the number cells. The surviving fraction in the treated wells was subsequently calculated as the ratio of the number of clones counted over the number of cells plated corrected with the appropriate plating efficiency.

Cell cycle and apoptosis assays. Cell cycle distribution and apoptosis were determined in 22Rv1 cells. The cells treated with PBOX-15 alone were exposed to the compound for 24 hrs, after which the compound was removed and fresh media added for an additional 24 hrs. Cells treated with radiation alone, received a 2 Gy single dose fraction and were allowed to recover for 24 hrs prior to analysis. Cells treated with the combined protocol were exposed to PBOX-15 for 24 hrs, the compound was removed and fresh media added prior to the delivery of a single 2 Gy dose fraction. The cells were allowed to recover for 24 hrs prior to analysis. Cell cycle distribution was analysed by flow cytometry analysis of propidium iodide (PI; Invitrogen) stained cells. Following treatment with PBOX-15 and/or radiation (2 Gy), as above, cells were fixed and permeabilised in 90% (v/v) ethanol, and incubated with 25 μ g/ml PI and 0.1 mg RNase A (Gentra Systems Inc., Minneapolis, MN) for 30 min prior to analysis using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, Franklin Lakes, NJ). The induction of caspase-mediated apoptosis in cells treated with PBOX-15 and/or radiation (2 Gy) was evaluated using a Caspase-Glo[®] 3/7 assay kit (Promega, Madison, WI) as per manufacturer's instructions.

RNA isolation and quantitative RT-PCR analysis. Quantification of *HIF-1 α* mRNA levels was performed in duplicate by a real-time fluorescence detection method as described previously in reference 51. In brief, after RNA isolation with an RNeasy Mini Kit (QIAGEN, Valencia), 2 μ g of total RNA was converted to cDNA with a first strand High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Ltd., Warrington, Cheshire, UK). The HIF-1 α cDNA and internal control cDNA (*PGK1*) were PCR-amplified separately (Applied Biosystems). Relative gene expression was determined by the threshold cycles for the *HIF-1 α* gene and the *PGK1* gene. A threshold relative induction of 2-fold was used.

Western blot analysis. HIF-1 α protein expression was determined in whole cell lysates of aerobic and hypoxic 22Rv1 cells treated with PBOX-15. The cells were scraped under hypoxic conditions and stored on ice. The pellet was resuspended in lysis buffer.⁵² Protein was extracted, subjected to polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes (Amersham, Little Chalfont, UK) were then probed with mouse monoclonal anti-HIF-1 α antibody (1:250, BD Biosciences, UK) and Horseradish peroxidase conjugated rabbit anti-mouse secondary antibody (1:1,000, Amersham, UK). SuperSignalWestPico Chemiluminescence substrate (Pierce, Northumberland, UK) was used for protein detection. Membranes were stripped prior to reprobing with a mouse monoclonal anti-actin antibody (1:10,000, Sigma-Aldrich, Poole, UK).

Statistical analysis. All experiments were performed in triplicate. Differences in surviving fraction, % cells, apoptosis levels and relative gene expression were compared using Student's t-test or Analysis of Variance (ANOVA) where appropriate. A p value of <0.05 was considered statistically significant. Data are presented as Mean \pm Standard Error of the Mean.

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