Using the conserved hexapeptide in HOX proteins as an antagonist of HOX/PBX interactions

Using the conserved hexapeptide in HOX proteins as an antagonist of HOX/PBX interactions

Running title: Peptide-based inhibition of HOX/PBX interactions

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Abstract

The HOX and PBX genes encode transcription factors that have key roles in development and cancer, both independently and as a heterodimer within a complex of proteins that recognizes specific sequences in DNA and can both activate or repress transcription of target genes. Due to functional redundancy amongst HOX proteins, knock down or knock out studies of individual genes often do not result in an altered phenotype. An alternative approach is to target the interaction between HOX and PBX proteins, which is dependent on a conserved hexapeptide region within HOX. To this end, several peptides have been developed based on the hexapeptide sequence which act as competitive antagonists of HOX/PBX binding, including HXR9 and HTL001. Here, we review the methodology that has been used in these studies, including peptide syntheses, cell culture, assays, and mouse models.

1 Introduction

1.1 HOX and PBX genes

The HOX genes are a family of homeodomain-containing transcription factors that have key roles in the determination of cell and tissue identity, which begin during gastrulation and continue into adulthood ***(1)***. They are present in 4 distinct chromosomal clusters (A-D) in which they share enhancer regions, conferring both temporal and spatial regulation of their expression, which in turn underpins early patterning events ***(1)***. The binding affinity and specificity of HOX proteins for DNA is relatively low, but is increased many fold by binding to

cofactors including PBX proteins, and interactions between HOX and PBX proteins is an important component of the molecular control of developmental pathways ***(2)***.

Over the last few decades, it has become increasingly apparent that both HOX and PBX genes also have an important role in cancer and are frequently overexpressed in tumors. Although both tumor suppressor and oncogenic functions have been described for HOX genes, the great majority of studies that assess the function of HOX proteins (as opposed to associate studies) indicate that their role is primarily oncogenic ***(3)***. Likewise, PBX genes have frequently been ascribed oncogenic functions ***(4)***.

1.2 Targeting the interaction between HOX and PBX

The multiplicity of HOX genes that have high levels of sequence similarity and correspondingly high levels of functional redundancy make it unlikely that targeting a single HOX gene in cancer will be a successful therapeutic strategy, and indeed this has proved to be the case in several previous studies ***(5)***. An alternative strategy is to target the interactions between HOX proteins and their cofactors in order to disrupt the multi-component protein complexes of which they form part. To date, the most widely reported approach is to use the hexapeptide region of HOX proteins as a competitive antagonist of the HOX/PBX interaction ***(5)***. The first publication on the use of such an antagonist in 2007 described a synthetically produced peptide, HXR9, that consists of the conserved hexapeptide region of HOX proteins joined to a poly-arginine region (R9) that promotes uptake of the peptide by cells, most likely by endocytosis ***(6)***. This mechanism of uptake is supported by the rapid entry into cells in vitro. This study used an immunoprecipitation assay to demonstrate that HXR9 could disrupt the interaction between HOXD10 and PBX1 in melanoma cells – an anti-PBX1 antibody could pull down HOXD10 from cell lysates in untreated cells, but not in cells treated with HXR9. As a control, a peptide differing from HXR9 by just a single amino acid was used – CXR9. A tryptophan residue was previously shown to be a key requirement for binding to PBX, and in CXR9 this is replaced by alanine. Unlike HXR9, CXR9 cannot block the immunoprecipitation of HOXD10 by an anti-PBX1 antibody. As an additional control, a second binding partner of PBX, glucocorticoid receptor (GR) was also immunoprecipitated from cells using the anti-PBX1 antibody. GR interacts with PBX through a different, hexapeptide-independent site, and correspondingly it was shown that HXR9 did not disrupt PBX1/GR binding, indicating that HXR9 specifically

antagonizes the interaction between PBX1 and HOX proteins. This study went on to show that HXR9 could trigger apoptosis in melanoma cell lines and primary cells, and significantly reduce the growth of murine B16F10 tumors in vivo. The mechanism of action seemed to be a rapid increase in the expression in several genes that had previously been identified as HOX targets, most notably cFos, knock-down of which with antisense oligos could partially rescue cells form HXR9-induced apoptosis ***(6)***.

Since the publication of this study, there have been a multiplicity of other studies in a range of cancers by different groups, all of which show that HXR9 could cause apoptosis in cancer cells in vitro, and in some cases reduce tumor growth in vivo. These include prostate ***(7)***, renal ***(8)***, breast ***(9)***, lung ***(10)***, and esophageal cancer ***(11)***, mesothelioma ***(12)***, chordomas ***(13)***, glioma ***(14)***, meningioma ***(15)***, and acute myeloid leukemia (AML) ***(16)***. In addition to expanding the range of cancers, this subsequent work has also provided a far clearer understanding of the mechanism of action of HXR9. Most of the genes that undergo rapid transcriptional changes in response to HOX/PBX inhibition can be classified as "early growth response genes" that are often upregulated in response to cellular stress. This might imply an indirect form of regulation, whereby peptides such as HXR9 induce a stress response, which in turn triggers apoptosis. However, there is growing evidence that these genes are indeed direct targets of HOX/PBX regulation. The majority of HXR9 target genes have HOX/PBX binding consensus sites in their promoter or enhancer region ***(9)***, this includes miR221 and miR222 ***(17)*** and p53 ***(9)***. This regulation of early response genes by HOX/PBX dimers reflects well characterized events in the developing embryo and in the differentiation of adult stem cells where HOX genes have a key role in defining the identity of multiple cells and tissues. Most notably, EGR2 (krox20), which is strongly upregulated by HXR9 treatment in lung cells ***(10)***, is specifically expressed in the 3rd and 5th rhombomeres of the developing hindbrain ***(18)***. Early on in development krox20 expression in the hindbrain is driven by HOX/PBX dimers binding to the promoter region of this gene, but this is subsequently inverted as the brain develops, with HOX/PBX dimers inhibiting krox20 expression ***(18)***. Similarly, EGR1 (Krox-24), which is required for the inhibition of granulocytic differentiation and the promotion of monocytic differentiation in hematopoietic progenitor cells, is regulated by HOXB8 ***(19)***.

1.3 Assays for HOX/PBX inhibition

To date, the only means of directly demonstrating the disruption of HOX/PBX interactions is through immunoprecipitation of PBX1 which pulls down a HOX protein in untreated cells, but not in peptide treated cells. Inhibition can also be measured indirectly by following a number of characterized changes in cells that occur almost invariably after treatment, often within just a few hours. Although none of these changes are specific to HOX/PBX inhibition, they can make useful surrogate markers for this when direct inhibition has also been demonstrated. These include apoptosis and changes in the expression levels of the specific downstream target genes described above. The methodology for performing these assays is therefor included in this chapter.

2 Materials

2.1 Cell culture

All cell lines can be sourced from the ATCC. Cells maintained at 37°C with 5% CO_2 unless specified.

2.2.1

Caki-2 cells, from renal clear cell carcinoma, culture in McCoy's 5A medium supplemented with 1% penicillin/streptomycin, 10% heat inactivated fetal bovine serum and 1.5 mM L-glutamine (Gibco®) ***(8)***.

2.2.2

769-P cells, from renal clear cell carcinoma, culture in RPMI-1640 medium supplemented as described for Caki-2 except 2 mM L-glutamine ***(8)***.

2.2.3

A549 cells, from epithelial lung carcinoma, culture in F-12K Nutrient Mixture Kaighn's Modification medium supplemented with 10% fetal calf serum and 1% penicillin (10 000 U/ml) / streptomycin (10 mg/ml) ***(10)***.

2.2.4

H23 cells, from non-small cell lung cancer, adenocarcinoma, culture in F-12K Nutrient Mixture Kaighn's Modification medium supplemented with 10% fetal calf serum and 1% penicillin (10 000 U/ml) / streptomycin (10 mg/ml) ***(10)***.

2.2.5

B16F10 cells, from mouse melanoma, culture in RPMI medium supplemented with 10% fetal calf serum, 2 mmol/L of L-glutamine, and 1% penicillin/streptomycin, in a 10% CO_2 incubator ***(6)***.

2.2.6

SK-BR-3, from breast adenocarcinoma, culture in McCoy's 5A medium supplemented with 10% heat inactivated fetal bovine serum ***(9)***.

2.2.7

MDA-MB-231 cells, from breast adenocarcinoma (epithelial), culture in Leibovitz's L-15 medium supplemented with 10% heat inactivated fetal bovine serum ***(9)***.

2.2.8

MCF7 cells, from breast adenocarcinoma (epithelial), culture in Eagle's Minimum Essential medium supplemented with 0.01 mg/ml human recombinant insulin and 10% fetal bovine serum ***(9)***.

2.2.9

ZR-75-1 cells, from breast ductal carcinoma (epithelial), culture in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum ***(9)***.

2.2.10

UACC-812 cells, from breast ductal carcinoma (epithelial), culture in Leibovitz's L-15 medium supplemented with 2 mM L-glutamine, 20 ng/ml human EGF, and 20% fetal bovine serum. Note: air only (no CO_2) ***(9)***.

2.2.11

MCF10a cells, from breast, fibrocystic disease (epithelial), complex culture conditions: use MEGM kit catalogue number CC-3150, Lonza/Clonetics Corporation ***(9)***.

2.2.12

U87-MG cells, from a probable glioblastoma, epithelial, culture in Eagle's Minimum Essential medium supplemented with 10% fetal bovine serum ***(14)***.

2.2.13

HT-29 cells, from colorectal adenocarcinoma, culture in McCoy's 5A medium supplemented with 10% heat inactivated fetal bovine serum ***(14)***.

2.2.14

LN-18, from epithelial glioblastoma, culture in Dulbecco's Modified Eagle's medium supplemented with 5% heat inactivated fetal bovine serum ***(14)***.

2.2.15

DU145, from prostate carcinoma, epithelial, brain metastasis, culture in Eagle's Minimum Essential medium supplemented with 10% heat inactivated fetal bovine serum ***(7)***.

2.2.16

PC3 cells, from prostate adenocarcinoma, epithelial, bone metastasis, culture in F-12K medium supplemented with 10% heat inactivated fetal bovine serum ***(7)***.

2.2.17

LNCaP clone FGC, from metastatic prostate carcinoma, lymph node aspirate, culture in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum ***(7)***.

2.2.18

WPMY-1 cells, from Myofibroblast cells from normal prostate stroma, culture in Dulbecco's Modified Eagle's medium supplemented with 5% heat inactivated fetal bovine serum ***(7)***.

2.2.19

Met-5a cells, from normal mesothelium epithelial cells, complex culture requirements – please see ATCC for details ***(12)***.

2.2.20

NCI-H28 cells, from mesothelioma, culture in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum ***(12)***.

2.2.21

MSTO-211H cells, from biphasic mesothelioma, culture in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum ***(12)***.

2.2.22

NCI-H2052 cells, from mesothelioma, epithelial morphology, culture in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum ***(12)***.

2.2.23

NCI-H226 cells, from mesothelioma, squamous cell carcinoma, culture in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum ***(12)***.

2.2.24

SK-OV-3 cells, from ovarian adenocarcinoma, culture in McCoy's 5A medium modified, supplemented with 10% heat inactivated fetal bovine serum ***(20)***.

2.2.26

OV-90 cells, from malignant papillary serous adenocarcinoma, culture in 1:1 mixture of MCDB 105 medium containing 1.5 g/L sodium bicarbonate and Medium 199 containing 2.2 g/L sodium bicarbonate, supplemented with 15% heat inactivated fetal bovine serum ***(20)***.

3 Methods

3.1 Peptide sequence and synthesis

3.1.1

HXR9

Sequence: WYPWMKKHHRRRRRRRRR, inhibits HOX/PBX interactions ***(6)***.

3.1.2

CXR9

Sequence: WYP**A**MKKHHRRRRRRRRR, control peptide (note single amino acid difference with HXR9) ***(6)***.

3.1.3

HTL001

Sequence: WYKWMKKAARRRRRRRRR, inhibition of HOX/PBX interactions, shown to have increased efficacy with respect to cell killing ***(14)***.

3.2

Peptide synthesis

For use in cell culture these peptides are usually synthesized with no modifications (l-isomer), typically with a purity of >90%. For use in vivo, the peptides are synthesized with d-isomers at the N- and C-terminal amino acids to increase their half-life – note that for consistency the same batch of peptide should be used for the cell culture work and mouse models if part of the same study. For most of the studies referenced here a total of around 500 mg of peptide would have been used.

3.3

Peptide Reconstitution

All of the peptides listed in 3.1 are highly soluble in water (up to 200 mg/ml) and are thus soluble in all commonly used aqueous buffers. Provided below is a protocol for the reconstitution of the peptides from lyophilized powder.

3.3.1. Equilibrate HTL-001/HXR9/CXR9 lyophilized powder and sterile PBS to room temperature.

3.3.2. If required, sterilize the measuring utensils in 70% ethanol for 15 minutes.

3.3.3. Weigh desired amount of powder into a sterile bijou or an alternative sterile container.

3.3.4. Add a pre-calculated volume of sterile PBS dropwise directly to the weighed powder in the container, replace lid and leave for 10 minutes at room temperature to fully reconstitute.

3.3.5. Check that the powder has fully reconstituted, if not leave for an additional 10 minutes. (Check no powder remains up the side of the tube).

3.3.6. Lightly vortex bijou so the solution is uniform in color.

3.3.7 Aliquot as desired and store aliquots at -80°C. (Excess powder may be stored at -20°C).

3.4 Cell culture

Cell culture for HOX/PBX inhibition studies is conducted based on the specific requirements of cell lines used (2.1).

3.5 Peptide treatment

Inhibitory (HXR9 and HTL-001) and inactive control (CXR9) peptides are synthesized and prepared as described in section 3.1. For gene expression and cell survival assays, generally peptide treatment of cells in culture is for a duration of 2 hours when cells have reached 80% confluence, with concentration increasing stepwise from 10 μM to 200 μM.

3.6 Peptide uptake into cells

Peptide uptake into cells is typically assayed using peptides with N- or C-terminal fluorescent tags, most typically FITC. Uptake is primarily through endocytosis and consequently occurs quite rapidly ***(21)***, and treatment durations are correspondingly shorter than for other assays (section 3.3), typically 10 to 30 minutes, using a low concentration of peptide (5 μM in most studies). Cells are counterstained with DAPI (blue florescence) to show the cell nucleus.

3.7 RNA extraction and RT-QPCR

RNA extraction and RT-QPCR are used in most studies to examine changes in expression of HOX/PBX target genes. Typically, the protocol is as follows:

3.7.1 Grow cells in sufficiently large flasks to allow the retrieval of around 1×10^6 cells, begin treatment at 70-80% confluency as described above (section 3.3)

3.7.2 After treatment dispose of the media and wash cells with 5 ml cold PBS and ensure that as much of the remaining fluid as possible is removed

3.7.3. RNA extraction is achieved using the Qiagen RNeasy mini kit. To 1 ml of extraction buffer RTL add 10 μL of beta-mercaptoethanol and mix. Add to the flask and remove the cells using a cell scraper.

3.7.4 Take 350 μL of the resulting cell lysate and add 350 μL 70% ethanol and mix by pipetting in a 1.5 ml Eppendorf tube.

3.7.5 Add the mixture to a RNeasy® spin column and place in a 2 ml collection tube, spin for 15 seconds at 10,000 g and discard the flow through.

3.7.6 Add 350 μL of RW1 buffer to the spin column and repeat the centrifuge step, again discarding the flow through.

3.7.7 Prepare a DNase I stock solution by adding 10 μL of DNaseI to 70 μL of buffer RD1 and pipette onto the membrane of the spin column, incubate the column at room temperature for 15 minutes.

3.7.8 Repeat the RW1 wash step and discard the flow through.

3.7.9 Add 500 μL of buffer RPE and centrifuge for 15 seconds at 10,000 g, discard the flow through and collection tube.

3.7.10 Place the spin column in a new 2 ml collection tube and spin for 2 minutes at 10,000 g, discard the flow through and collection tube.

3.7.11 Place the spin column in a 1.5 ml tube and pipette 30 μL of molecular biology grade water onto the membrane, leave at room temperature for 2 minutes.

3.7.12 Centrifuge for 2 minutes at 10,000 g and discard the spin column.

3.7.13 Measure the RNA concentration along with the A260/280 and A240/260 ratios using a nanospectrophotometer.

3.7.14 For cDNA synthesis a range of kits can be used. For the High Capacity RNA to cDNA kit (Thermofisher), use the following steps:

3.7.15 Mix 10 μL of 2RT buffer and 2 μL of 20RT enzyme mix with molecular biology grade water and 1 μg of RNA in a total volume of 20 μL.

3.7.16 Incubate at 37°C for 30 minutes, followed by 95°C for 5 minutes.

3.7.17 Cool the tube to room temperature and centrifuge briefly to collect the fluid at the bottom of the tube.

For quantitative PCR of target genes, the majority of studies have used a SYBR green based approach.

3.8 Immunofluorescence

Precise conditions for immunofluorescence need to be determined for different antigens / antibodies. For HOXA9 expression in HTL001-treated cells the following method has been successfully used:

3.8.1 Place a 22 mm × 22 mm cover slip in each well of a 6-well plate and seed with 8×10^4 cells in 2 ml media, incubate at 37°C with 5% CO_2.

3.8.2 Once the cells have reached 70-80% confluency (usually after 24 hours) treat with 28 µM or 94 µM HTL001 or CXR9 in media for two hours or with different concentrations of the control peptide, for two hours.

3.8.3 Wash the cells 3 times with PBS without disrupting them, fix by adding 1 ml of pre-cooled methanol and keep at -20°C for 10 minutes.

3.8.4 Remove the methanol and dry the plate dried in a class II cabinet for 30 minutes.

3.8.5 Rehydrate cells with 2 × 1 ml washes with PBS, then permeabilize with 0.1% Triton X-100 solution.

3.8.6 Non-specific binding of antibodies is blocked by incubation for 1 hour at room temperature with 1 ml 5% BSA in PBS.

3.8.7 To each cover slip add 200 µl of 1:100 dilution of primary anti-HOXA9 rabbit polyclonal antibody (Abcam, UK), incubate overnight at 4°C.

3.8.8 Wash the cells three times with 1 ml PBS for 5 minutes.

3.8.9 Prepare a 1:400 dilution of the secondary antibody, goat anti-rabbit IgG (H&L) (Alexa Fluor® 488, Abcam, UK) in PBS, add 500 µl to each well incubate for 1 hour in the dark at room temperature.

3.8.10 Wash the cover slips again with 1 ml PBS, 3 × 5 minutes.

3.8.11 Remove the cover slips from the wells and place cell-side down on a microscope slide with 25 µl of Vectashield® hard set antifade mounting media with DAPI.

3.8.12 Incubate in the dark for a further 30 minutes at 4°C.

3.8.13 Examine the cells using a fluorescence microscope; for DAPI (blue) the excitation is at 358 nm and the emission at 461 nm, for FITC (green) the excitation is at 490 nm and the emission at 461 nm.

3.9 Band shift assays

Band shift assays are an important tool for demonstrating specific interactions between proteins and DNA, particularly within the context of HOX/PBX inhibition studies, to demonstrate disruption of HOX/PBX/DNA complexes.

Cell lysates for the band shift assay are made from cells or tumors treated with HXR9/HTL001/CXR9 as described in sections 3.3 and 3.12. Lysates are produced using commercially available kits, following the manufacturer's guide and modified depending on cell type.

For band shift of sequences containing the HOX/PBX binding consensus site, the following method can be used:

3.9.1 Synthesize double stranded DNA probes - the forward sequences of these oligos are: HP (containing a HOX/PBX consensus binding site)—GGACA AACTG AAGGCAGAGCTGATT TATGG CACAC ACACA AGAAT GGACA AACCC GTGAG and HPC (containing an altered HOX/ PBX binding site as a control)—GGACA AACTG AAGGC AGAGC GCTCC GTTAA CACAC ACACA AGAAT GGACA AACCC GTGAG. The oligos are linked to streptavidin are the 5' end.

3.9.2 Make a 1:1 mixture of the forward and reverse oligos for each probe at a concertation of 10 µM in a buffer containing 1 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-EDTA pH 8.0. Heat to 90°C for 5 minutes and then allow to cool slowly to room temperature.

3.9.3 Add 2 µL of HP or HPC to 100 µL cell lysate and incubate at room temperature for 1 hour.

3.9.4 Use a streptavidin column to retrieve the DNA probes and resolve using non-denaturing PAGE.

3.9.5 Transfer to a PVC membrane and use anti-HOX or anti-PBX antibodies to confirm HOX/PBX binding to shifted probe.

3.9.6 For super shift experiments, use an anti-PBX1,2,3 antibody (c-20; Santa Cruz Biotechnology) at a dilution of 1:2,000.

3.10 Immunoprecipitation

Immunoprecipitation can also be used to demonstrate the disruption of HOX/PBX complexes. Several similar methods have been described, but the most commonly followed is for HOXB7/PBX2 (Figure 1) ***(17)***:

3.10.1 After treatment, fix 3×10^6 A375M melanoma cells in 1% formaldehyde for 10 min at room temperature.

3.10.2 Wash cells with ice cold PBS and collect by scraping with cold PBS plus protease inhibitors and collect by centrifugation.

3.10.3 Resuspend cell pellets in cell lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) in the presence of protease inhibitors, and sonicate.

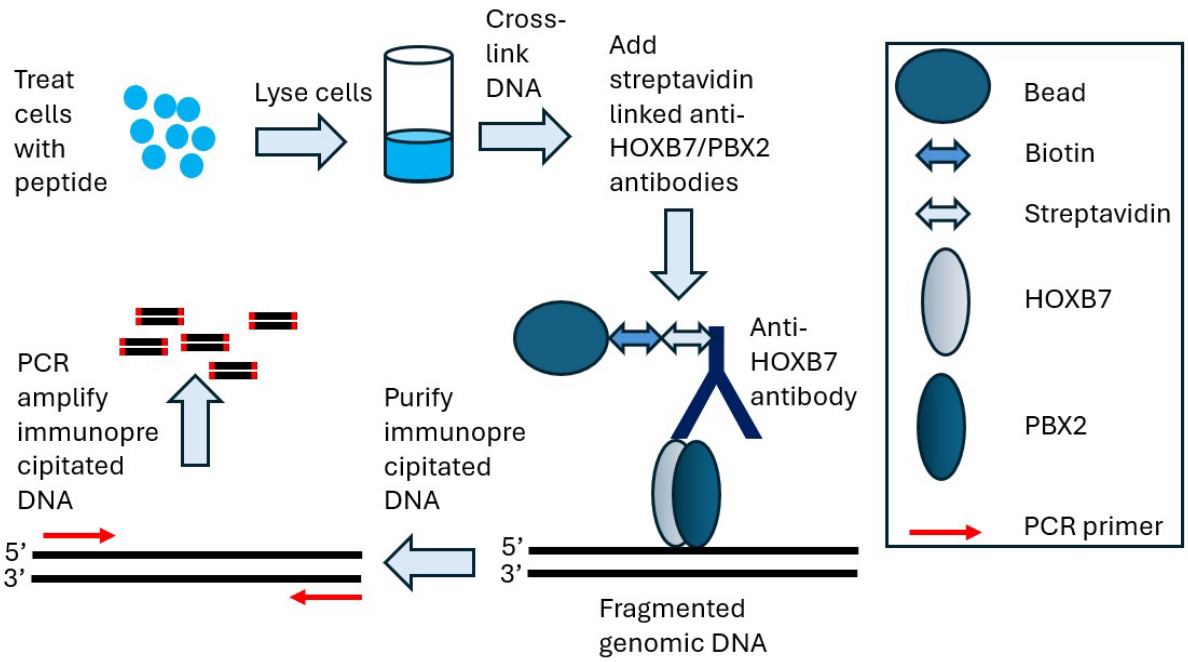
3.10.4 DNA-protein complexes can be immunoprecipitated using anti-HOXB7 (Invitrogen), anti-PBX2 or, as an internal control, the unrelated anti-DVL-1 (Santa Cruz Biotechnology).

3.10.5 Reverse DNA-protein crosslinks by heating at 65°C overnight.

3.10.6 The DNAs recovered in this process are then PCR amplified with the following primers: Forward 5’-CAGCATACATGATTCCTTGTGA-3’ and Reverse 5’-CTTTGGTGTTTGAGATGTTTGG-3’, and Forward 5’-CCCACCAAGAGCTAACCACA-3’ and Reverse 5’-TCGGACATGCAGCTATACCA-3’, both of which amplify regions containing a HOX/PBX binding site. In addition, two negative control regions (lacking a HOX/PBX binding site) are also amplified.

3.10.7 In order to confirm the specificity of these PCR products PCR amplification should also be preformed on an unrelated DNA sequence, for example GAPDH or Beta actin.

Figure 1 Scheme summarizing the methodology used in PCR-based immunoprecipitation for HOXB7/PBX2 targets.



3.11 Apoptosis

Apoptosis is the usual mode of cell death after treatment of cancer cells with HOX/PBX inhibitors. A range of methods are suitable, but typically these are flow cytometry with Annexin or ELISA / western blot detection of caspase activation. Furthermore, the Incucyte Apoptosis Assay can be employed to measure apoptosis in real-time via multiple apoptotic pathways (Annexin V and Caspase-3/7).

For flow cytometry, a generally used method is to harvest treated cells by incubating in trypsin-EDTA at 37°C until detached and dissociated and then identify apoptotic cells using flow cytometry and the Annexin V-PE apoptosis detection kit (BD Pharmingen) as described by the manufacturers protocol.

For caspase activation, measure Caspase-3 activity using the EnzCheck Caspase-3 Assay Kit (Molecular Probes), using the protocol defined by the manufacturer.

For use with the Incucyte Live-Cell Analysis system, a protocol is provided below

3.11.1 For adherent cell lines, seed 100 µl of cells per well into a 96-well plate at a concentration to ensure 30% confluency the next day.

3.11.2 For non-adherent cell lines, precoat plates with 50 µl 0.01% poly-L-ornithine and leave for 1 hour at room temperature. Following incubation remove the coating matrix and leave the plates to dry for 30 minutes before the addition of the cell seeding solution.

3.11.3 Leave the cells in the incubator overnight to attach.

3.11.4 The next day, dilute the Annexin V dye 1:200 in complete medium containing 1mM CaCl2, and dilute the caspase-3/7 green dye 1:1000 in complete medium to achieve a final assay concentration of 5 µM.

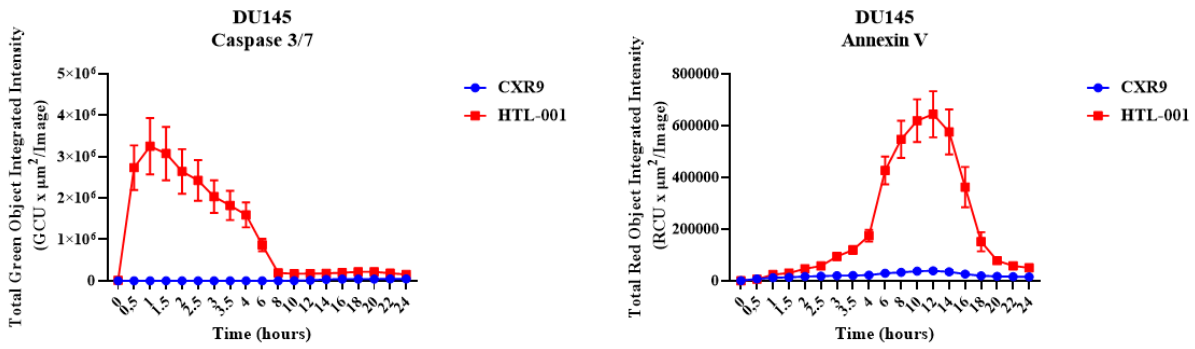
3.11.5 Remove the plates from the incubator and carefully aspirate the culture medium.

3.11.6 Replenish wells with fresh culture medium containing diluted caspase-3/7 and Annexin V dyes and the appropriate treatment or control.

3.11.7 Place the cell plate into the Incucyte Live-Cell Analysis System to monitor apoptosis in real-time using phase contrast and fluorescence. Due to the rapid nature of the HTL-001 cytotoxicity, include the capture of images at regular intervals for the first 4 hours of treatment (e.g., every 10-30 minutes) and then every few hours thereafter up to 24 hours.

An example of cell growth data obtained using Incucyte is shown in Figure 2.

Figure 2 The Incucyte Live Cell Analysis System and Apoptosis Assay to assess caspase-dependent apoptosis through capase-3/7 and Annexin V. DU145 prostate adenocarcinoma cells were treated with an IC50 dose of HTL-001 or an equivalent dose of CXR9. Graphs show caspase-3/7 and Annexin V activity throughout the 24-hour assay, with each data point the mean of three independent experiments and the error bars represent the standard error of the mean. Brightfield and fluorescence images (10× magnification) were captured every 30 minutes for the first 4 hours and then every 2 hours thereafter until the end of the assay.



3.12 Mouse models

Unless specified, treatment is started when tumors reach a volume of 100 mm^3 (day 1), using athymic nude mice. Duration of treatment refers to the maximum length of treatment, mice were euthanized if showing signs of distress per individual institutional guidelines.

3.12.1

Mesothelioma

This is established with a flank injection of 2.5 x 10^6 MSTO-211H cells (2.2.21), with IP delivery of 25 mg/kg HXR9 every 4 days, up to a maximum of 36 days after the initial treatment ***(12)***.

3.12.2

Non-small cell lung cancer

Established with a flank injection of 2.5 x 10^6 A549 cells (2.2.3), with an initial dose of HXR9 or CXR9 100 mg/kg IP or IT, subsequent dosing 10 mg/kg twice weekly, tumors are not allowed to exceed 400 mm^3 ***(10)***.

3.12.3

Murine melanoma

B16F10 cells (1.0 × 10^6) (2.2.5) are injected into the flank of adult C57/black 6 mice. Treatment is 10 mg/kg HXR9 twice weekly IV with a maximum tumor volume of 1200 m^3 ***(6)***.

3.12.4

Breast cancer

MDA-MB-231 cells (2.5×10^6) (2.2.7) are injected into the flank and tumors are treated with 100 mg/kg HXR9 IT on day 1, subsequent treatment if tumors regrow for a maximum of 50 days.

3.12.5

Ovarian cancer

SK-OV-3 cells (2.5×10^6) (2.2.24) injected into the flank, treatment consists of an initial dose of HXR9 or CXR9 100 mg/kg IV with subsequent dosing 10 mg/kg twice weekly for a maximum of 35 days ***(20)***.

3.12.6

Glioblastoma

Flank injection of 1.0×10^6 U87-MG cells (2.2.12) followed by treatment of 30 mg/kg HTL001 or CXR9 IP on day 1 (when tumors are 80-90 mm^3) and then max 3 times / week for 3 weeks ***(14)***.

3.12.7

Orthotopic model of glioblastoma

Induce intracranial tumors in C57BL/6 J mice by injecting GL261 cells (1×10^5) in a total volume of 2 μl into the striatum of mice by stereotactic injection, and randomly assign to control and treatment groups. Seven days after tumor inoculation, the tumor-bearing mice are treated IP with CXR9 or HTL001 (30 mg/kg) three times a week until the end of the experiment or, for IT treatment, 600 μg of CXR9 or HTL001 in 5 μl PBS are injected into the tumor. Tumor growth is monitored using MRI with a 7 Tesla MRI scanner (Bruker Biospin) ***(14)***.

3.13

Distribution of peptide after treatment

3.13.1 In order to follow the distribution of peptides, HTL-001 labelled with Alexa 660 is administered IP ate 10 mg/kg into tumor-bearing mice. After 30 minutes the mice are sacrificed and organs harvested for imaging using a Cy5.5 filter in the IVIS imaging system (Perkin Elmer) with an exposure time of 10 seconds.

3.13.2 For inter-cranial distribution, 6-8 week-old male or female NOD scid gamma mice are used. GBM12-RFP cells are implanted stereotaxically into the right hemisphere, and craniectomy is performed 2 weeks later in which a cover glass is fixed to the skull.

3.13.3 For intravital imaging, mice are anesthetized and positioned on the stage of a confocal microscope to acquire pre-treatment images. Subsequently, 100 µl of HTL-001-Alex 660 peptide (5 mg/ml in PBS) is administered IV or IP, followed after one hour by 100 µl of 10% FITC-conjugated dextran IV, post-treatment images are then acquired ***(14)***.

3.14 Immunohistochemistry

Immunohistochemistry is used in many studies on HOX/PBX inhibition to assess the protein expression of HOX or PBX proteins, or for putative downstream targets. Experimental details vary for different targets and tissues, but for HOXA4, HOXA9, and HOXB4 in mesothelioma and normal mesothelium tissue the following method is used:

3.14.1 Use 3 µm-thick, formalin fixed, paraffin embedded tissue array sections (US Biomax).

3.14.2 Immunohistochemical analysis is performed using a monoclonal rabbit anti-HOXB4 antibody (1:100 dilution, Abcam), a polyclonal rabbit anti-HOXA4 antibody (1:500 dilution, Abcam), and a polyclonal rabbit anti-HOXA9 antibody (1:75 dilution, Abcam).

3.14.3 For each of these primary antibodies, the ABC detection method with peroxidase block (DakoCytomation) is used and antigen retrieval is performed with pH 9.0 Tris/EDTA buffer (DakoCytomation) and heating in a microwave for 23 min.

3.15 Short interfering RNA (siRNA) knockdown of HOX and PBX genes

Knockdown of specific HOX and PBX genes has proven informative in several studies involving inhibition of HOX/PBX dimers, including HOXB7 and PBX2 ***(17)***:

3.15.1 After reaching confluency, cells are transfected using Fugene HD (Promega) with siRNA-HOXB7 (with three different sequences: HSC.RNAI.N004502.12.1, HSC.RNAI.N004502.12.2, and HSC.RNAI.N004502.12.3), siRNA-PBX2 (HSC.RNAI.N002586.12.1), or a scrambled control RNA (Dsi-scr #64218602), at a final concentration of 100 nM.

3.15.2 Levels of HOXB7 and PBX2 mRNA and protein are analyzed 48 hours after transfection using qRT-PCR and western blotting.

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