Biochemical and Biophysical Research Communications 453 (2014) 735–740

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Design of a PROTAC that antagonizes and destroys the cancer-forming X-protein of the hepatitis B virus

Kristopher Montrose, Geoffrey W. Krissansen *

Department of Molecular Medicine & Pathology, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand

ARTICLE INFO

Article history:
Received 16 September 2014
Available online 8 October 2014

Keywords:
Cell-penetrating peptide
X-protein
Proteolysis targeting chimeric molecule
Hepatitis B virus
Apoptosis

ABSTRACT

The X-protein of the hepatitis B virus (HBV) is essential for virus infection and contributes to the development of HBV-induced hepatocellular carcinoma (HCC), a disease which causes more than one million deaths each year. Here we describe the design of a novel PROTAC (proteolysis targeting chimeric molecule) capable of simultaneously inducing the degradation of the X-protein, and antagonizing its function. The PROTAC was constructed by fusing the N-terminal oligomerization and C-terminal instability domains of the X-protein to each other, and rendering them cell-permeable by the inclusion of a polyarginine cell-penetrating peptide (CPP). It was predicted that the oligomerization domain would bind the X-protein, and that the instability domain would cause the X-protein to be targeted for proteasomal degradation. Addition of the PROTAC to HepG2 liver cancer cells, engineered to express full-length and C-terminally truncated forms of the X-protein, resulted in the degradation of both forms of the X-protein. A cell-permeable stand-alone form of the oligomerization domain was taken up by HepG2 cells, and acted as a dominant-negative inhibitor, causing inhibition of X-protein-induced apoptosis. In summary, the PROTAC described here induces the degradation of the X-protein, and antagonizes its function, and warrants investigation in a preclinical study for its ability to prevent or treat HBV infection and/or the development of HCC.

1. Introduction

The ubiquitin–proteasome system is used by the cell to degrade and dispose of unwanted or misfolded cellular proteins [1]. The system targets proteins for proteasomal-mediated destruction by polyubiquitinating them in a process which involves the sequential action of the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin-protein ligase [1]. E3 ligase is responsible for attaching multiple 76 amino acid (aa) residue ubiquitin molecules to lysine residues at recognition sites in the target protein, which serve as a signal for proteolytic destruction by the 26S proteasome [1]. The ubiquitin–proteasome system has been exploited in the creation of proteolysis targeting chimeric molecules (PROTACs), which are engineered to target selected intracellular proteins for degradation [2–5]. PROTACs consist of a ligand capable of binding the target protein, fused to a peptide (referred to as the degron) that is recognized and polyubiquitinated by E3 ligase. Thus, the PROTAC non-covalently binds to a target protein, and recruits E3 ligase via the degron peptide, which results in polyubiquination and degradation of the bound target.

Commonly employed degrons include the oxygen-dependent degradation (ODD) domain of hypoxia inducible factor (HIF-1α), which is recognized by the von Hippel-Lindau (pVHL) E3 ligase [6,7], and a peptide from IκBα which is recognized by the β-transducin repeat-containing protein (β-TRCP) of the Skp1–Cullin–F box (SCF)$^{\beta\text{-TRCP}}$ E3 ligase complex [3]. Zhou and Howley pioneered PROTAC technology when they created a fusion peptide consisting of a subunit of SCF and the E7N binding partner of the phosphorylated retinoblastoma protein (pRb) [2]. The resulting peptide was able to cause the ubiquination and degradation of pRb *in vitro* [2]. The most common targets for therapeutic application of PROTACs to date have been cancer-associated proteins [8].

More than one-third of the world's population has been infected by the hepatitis B virus (HBV) [9]. The X-protein of HBV, which is essential for viral replication, is a ~154 aa residue protein of 17 kDa [10]. It is a major risk factor for the development of hepatocellular carcinoma (HCC) in patients chronically infected with

Abbreviations: aa, amino acid; CPP, cell-penetrating peptide; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HIF, hypoxia-inducible factor; ODD, oxygen-dependent degradation; pRb, retinoblastoma protein; PROTAC, proteolysis targeting chimeric molecule; SCF, Skp1–Cullin–F box; VHL, von Hippel-Lindau.

* Corresponding author.
E-mail address: gw.krissansen@auckland.ac.nz (G.W. Krissansen).

http://dx.doi.org/10.1016/j.bbrc.2014.10.006
0006-291X/

HBV [11–13]. The World Health Organization and the Centers for Disease Control and Prevention project that up to 87 million of the 350 million people chronically infected with HBV worldwide will eventually die of HCC [14]. The X-protein promiscuously transactivates and dysregulates multiple cancer-associated genes, DNA repair mechanisms and cell apoptosis pathways [10,11]. Removal of the C-terminal region spanning aa 141–154, which is responsible for the instability of the X-protein, leads to enhanced expression of the X-protein [15]. The ubiquitin–proteasome system is responsible for the degradation of the X-protein as proteasome inhibitors are able to increase the steady-state levels of the protein [16]. Regions of the X-protein that are polyubiquinated and susceptible to degradation lie within the middle of the protein at aa 52–102, and also within the instability domain at aa 103–154 [17]. Fusion of these regions to GFP rendered the fusion protein susceptible to proteasomal degradation, whereas the N-terminal domain which mediates dimerization of the X-protein [18], had little influence on ubiquitination or stability of the X-protein [17].

Here we report on the design and activity of a novel cell-permeable PROTAC which antagonizes and destroys the X-protein, and hence may have application in the treatment of HBV infection and/or prevention of HCC.

2. Materials and methods

2.1. Cells, peptides, and plasmids

The human HepG2 (liver cancer) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). It was propagated in MEM medium supplemented with 10% FCS and penicillin (100 U/ml), streptomycin (100 mg/ml), and L-glutamine (0.29 mg/ml) (PSG) at 37 °C and 5% CO_2. All peptides were synthesized by Peptide 2.0 Inc., Chantilly, VA. Peptide sequences were based on the consensus sequence of the X-protein of HBV prevailing in the mainland of China (Fig. 1A) [19]. Plasmid pcDNA3.1-HBX encoding a truncated form of the X-protein lacking the instability domain (aa 141–153) was prepared in-house by Adrina Khemlani. Plasmid pcDNA3-HBX Myc encoding a full-length form of the X-protein was kindly donated by Prof Massimo Levrero, Department of Internal Medicine, Universita di Cagliari, Italy.

2.2. Peptide binding assay to refine the oligomerization domain

Biotinylated X-protein peptide aa 1–50 (100 µl at 10 µg/ml) was added to the wells of a Reactibind neutravidin-coated 96-well plate (ThermoScientific), the plate incubated at room temperature for 2 h, and then washed. FITC-labeled truncated variants of peptide aa 1–50 were added at final concentrations of 10, 20 and 40 µg/ml in 100 µl of buffer (25 mM Tris–Cl, pH 7.2, 150 mM NaCl, 0.1% BSA, 0.05% Tween 20) to the peptide-coated wells, the plates incubated at room temperature for 30 min, washed, and fluorescence recorded on a Biotek fluorescence plate reader.

2.3. Assay of the cell-permeability of the oligomerization domain peptide

HepG2 cells were seeded into 8-well chamber slides at 1×10^5 cells per well in MEM medium (Gibco, Life Technologies New Zealand) containing 10% FCS and PSG. They were cultured overnight at 37 °C in a 5% CO_2 atmosphere, and washed thrice with serum-free MEM medium. The FITC-labeled oligomerization domain peptide was diluted in MEM medium without FCS, and added to cells at a final concentration of 10 µM. The cells were incubated for 3 h at 37 °C in a 5% CO_2 atmosphere, washed with PBS, fixed with 4% formaldehyde for 30 min, and washed thrice with PBS. A drop of Prolong Gold anti-fade reagent with DAPI (Invitrogen, Life Technologies New Zealand Ltd.) was added, and the cells mounted and examined with a Leica TCS-SP2 confocal microscope.

2.4. Transfection of HepG2 cells to express the X-protein

HepG2 cells were seeded into 6-well plates at 1×10^6 cells per well in MEM medium (Gibco, Life Technologies New Zealand Ltd.) supplemented with 10% FCS, and cultured overnight at 37 °C and

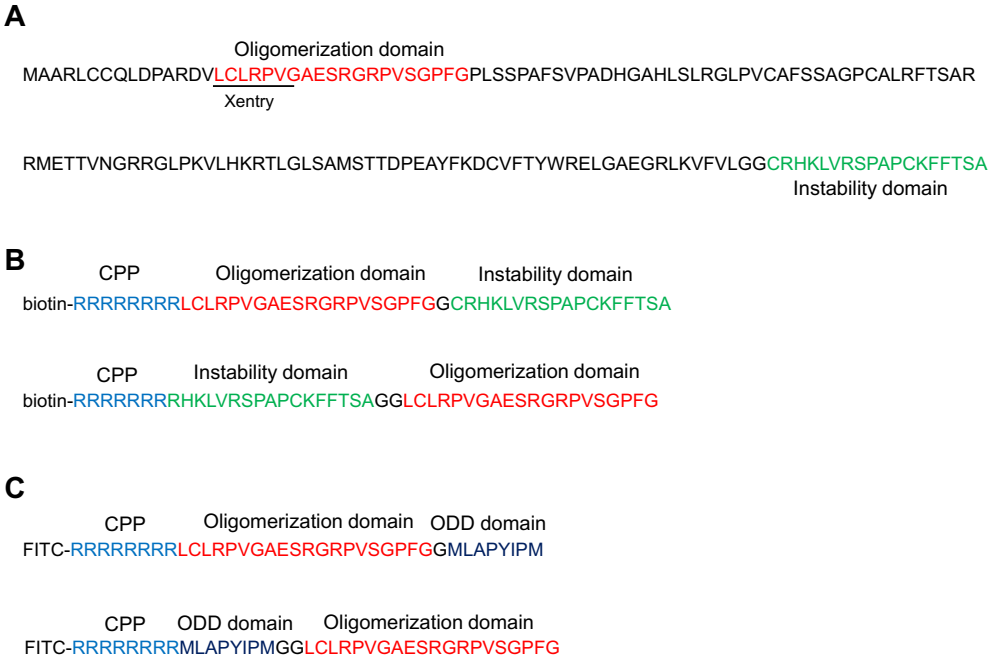

Fig. 1. (A) Sequence of the X-protein showing the positions of the oligomerization and instability domains, and the CPP Xentry. The sequence is the consensus sequence (GenBank accession number CAB38773, GenBank sequence database provided by the National Center for Biotechnology Information, Bethesda, MD) of the X-protein of HBV prevailing in mainland China, as reported by Guo and Hou [19]. (B and C) Sequences of the X-protein-targeting PROTACs based on the (B) instability domain of the X-protein, and (C) ODD domain of HIF-1α.

5% CO_2. The medium was replaced with MEM medium without FCS, and the cells were transfected with 4 μg of plasmids pcDNA3.1-HBX Myc and pcDNA3.1-HBX. The plasmids were mixed with 4 μl of polymag transfection reagent (OZ Biosciences, Marseille, France) in 250 μl of Opti-MEM media (Gibco, Life Technologies New Zealand Ltd.), and the mixture incubated for 20 min at room temperature and added to the cells. The tissue culture plate was placed on a rotating magnetic plate for 20 min. The medium was removed and replaced with MEM medium containing FCS, and the plates incubated at 37 °C and 5% CO_2 for 48 h with a change of medium after 24 h.

2.5. Analysis of X-protein degradation by the PROTACs

PROTACS based on the X-protein instability domain were synthesized with the X-protein oligomerization domain near the front, or end of the peptide (Fig. 1B). PROTACS based on the ODD domain of HIF-1α were similarly synthesized with the X-protein oligomerization domain near the front, or at the end of the peptide (Fig. 1C). The peptides were added to the above HepG2 transfectants at a final concentration of 10 μM in 1 ml of serum-free MEM medium, and the cells incubated at 37 °C and 5% CO_2 for 3 h followed by a change to serum-containing MEM medium, and further additions of peptide and changes of medium at 24 and 45 h. The cells were lysed 3 h after the final addition of peptide, and the lysates analyzed by Western blot analysis with a mouse anti-X-protein antibody (Chemicon, Temecula, CA). Blots were reprobed with a rabbit anti-human β-actin antibody (Sapphire Biosciences). Immunoreactivity was developed using horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies (Sigma, Mo), respectively, and visualized by enhanced chemiluminescence with a Fujifilm LAS 3000 scanner.

2.6. Analysis of the apoptosis of X-protein transfectants

Cell-permeable biotinylated peptides containing just the X-protein oligomerization domain (biotin-RRRRRRRRLCLRPVGA ESRGRPVSGPFG) or the instability domain (biotin-RRRRRRRRLVR SPAPCKFFTSA) as a control, which essentially represent the PROTACS devoid of either the degron peptide or target-binding domain, respectively, were synthesized. The peptides were repetitively added thrice (at 3, 24, and 48 h following addition of plasmid DNA) at a final concentration of 10 μM to HepG2 transfectants (1×10^4 cells per well) expressing the X-protein. The cells were cultured for a further 3 h, and apoptosis and necrosis were detected using an Annexin-V Fluos staining kit (Roche) with propidium iodide. The cells were washed, fixed with 4% formaldehyde in PBS for 30 min, a drop of Prolong Gold anti-fade reagent with DAPI was added, and the slide dried overnight in the dark. Slides were examined by microscopy using a Nikon E600 fluorescence microscope and photos were taken using Nikon ACT-1 software.

3. Results

3.1. Design of a PROTAC capable of degrading the X-protein

The X-protein naturally forms a dimer [20]. The oligomerization domain of the X-protein has previously been reported to lie within

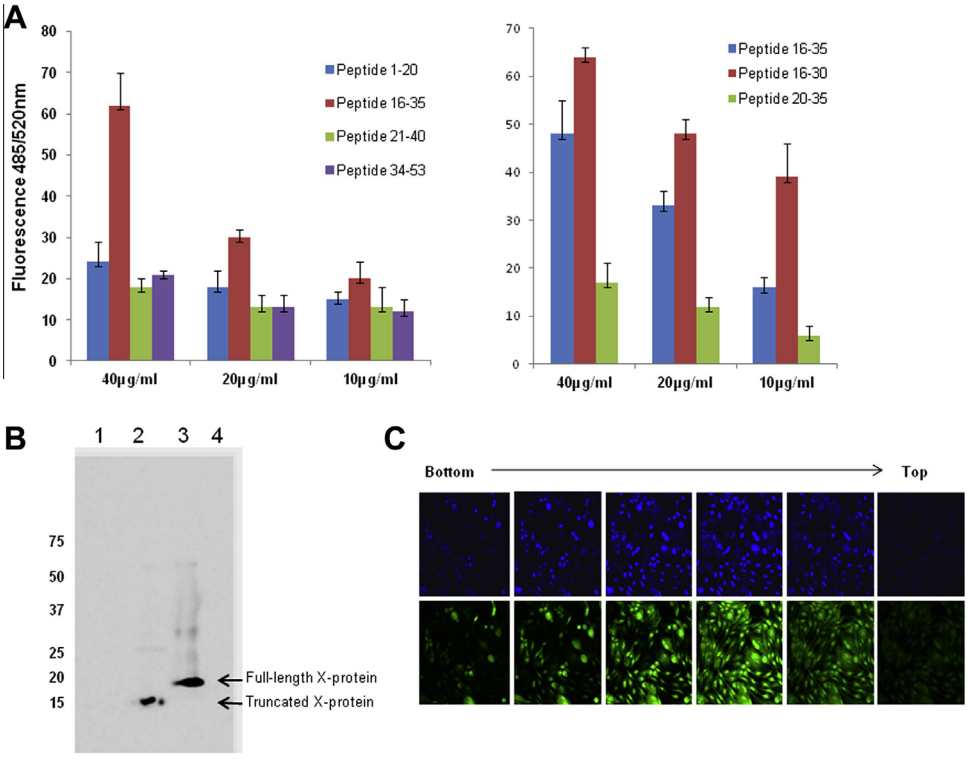

Fig. 2. (A) The oligomerization domain of the X-protein encompasses aa 16–30. FITC-labeled peptide fragments of the N-terminal region (aa 1–50) of the X-protein were tested for their ability to bind a biotinylated peptide encompassing aa 1–50 of the X-protein which had been bound to the wells of a neutravidin-coated plate. The fluorescence of the wells was recorded following peptide binding. (B) Western blot analysis of HepG2 cells engineered to express full-length and truncated forms of the X-protein. Detergent-insoluble extracts of HepG2 cells left untransfected (lane 1) or transfected with the plasmids pcDNA3.1-HBX (lane 2), pcDNA3-HBX Myc (lane 3), and a pcDNA3-GFP control plasmid (lane 4) were Western blotted with a mouse anti-X-protein antibody. Immunoreactivity was visualized using a goat-anti-mouse horseradish peroxidase conjugated antibody. The positions of the full-length (21 kDa) and truncated (17 kDa) X-proteins are indicated in the right-hand margin. Molecular weight markers are shown in the left-hand margin in kDa. (C) The oligomerization domain is cell-permeable. A FITC-labeled peptide encompassing aa 16–35 of the X-protein was incubated at 10 μM with HepG2 cells, and cell uptake recorded by confocal microscopy. Multiple optical slices of the cells were taken from the basal (bottom) to the apical (top) regions of the cells. Cell nuclei were stained blue with DAPI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

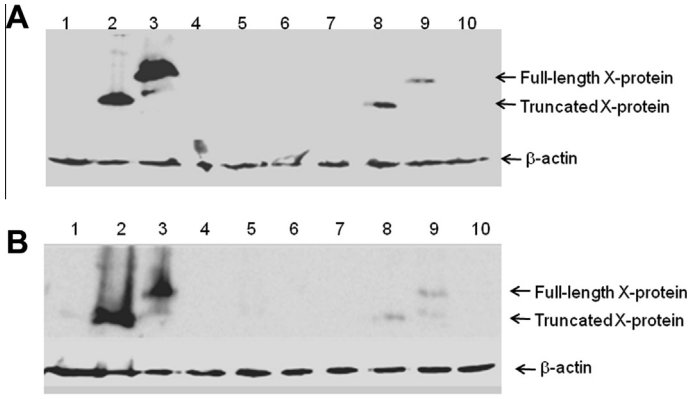

Fig. 3. PROTACs induce the degradation of the X-protein. (A and B) HepG2 cells were left untransfected (lane 1), or were transfected with plasmids encoding truncated (lanes 2, 5, and 8), and full-length (lanes 3, 6, and 9) forms of the X-protein, or were transfected with the control plasmid pcDNA3-GFP (lanes 4, 7, and 10). Transfectants were either left untreated (lanes 1–4) or were treated with the (A) PROTAC containing the X-protein instability domain located N-terminally (lanes 5–7) or C-terminally (lanes 8–10), or with (B) the PROTAC containing the HIF-1α ODD domain located N-terminally (lanes 5–7) or C-terminally (lanes 8–10). Detergent-insoluble extracts of the transfectants were subjected to Western blot analysis as described in Fig. 2B. The positions of the full-length and truncated forms of the X-protein, and β-actin which was used as a loading control, are shown in the right-hand margin.

the N-terminal region aa 1–50 [18]. A ligand-binding assay in which a biotinylated aa 1–50 peptide was bound to a neutravidin-coated plate and probed with short FITC-labeled peptides from the same region was undertaken to further refine the peptide sequence responsible for dimerization. The centrally located peptide aa 16–35 bound the parental peptide more strongly than peptides aa 1–20, 21–40 and 34–53 (Fig. 2A). Following truncation, a peptide containing aa 16–30 retained enhanced binding activity, whereas short peptides aa 20–35 (Fig. 2A), 16–26, 16–24, and 16–22 did not display enhanced binding activity (data not shown). A PROTAC was constructed by fusing the oligomerization domain (aa 16–35) of the X-protein to its instability domain (aa 140–154). Two versions of the peptide were created with the instability domain attached either in front or at the end of the oligomerization domain (Fig. 1B). The peptides were rendered cell-permeable by N-terminal fusion to a polyarginine CPP.

HepG2 cells were engineered to express the X-protein by transient transfection with plasmids encoding either the full-length wild-type X-protein or a C-terminally truncated X-protein lacking the instability domain (aa 141–153). An anti-X-protein antibody recognized the truncated (17 kDa) and full-length (21 kDa) forms of the X-protein present in the detergent-insoluble fractions of the transfectants (Fig. 2B). The transfectants were either left untreated or were treated with 10 μM of PROTAC at 3, 24 and 45 h post transfection. It was predicted that the PROTAC would be taken up by HepG2 cells, bind to the X-protein and target it for polyubiquitin-mediated degradation. The PROTAC with the instability domain in front caused complete degradation of both the full-length and truncated forms of the X-protein, whereas the PROTAC with the instability domain at the end was almost as effective, leaving only traces of the X-proteins (Fig. 3A). In contrast, β-actin, which served as a loading control was not affected by the PROTAC. These results establish that the X-protein instability domain can serve as a degron. Almost identical results were obtained when the X-protein instability domain was substituted with the ODD domain of HIF-1α (Fig. 3B).

3.2. The oligomerization domain of the PROTAC antagonizes the pro-apoptotic function of the X-protein

It was hypothesized that the PROTAC might disrupt the function of the X-protein by dual mechanisms; by inducing degradation of the X-protein as demonstrated above, and by acting as a dominant-negative inhibitor. The latter notion was based on the finding that the oligomerization domain transrepresses X-protein function [15,18], as evidenced by the reduction of X-protein-induced apoptosis of hepatocytes following retroviral expression of a peptide encompassing aa 1–78 [15]. A stand-alone version of the oligomerization domain (aa 16–35) of the X-protein lacking the degron peptide, which was rendered cell-permeable by fusion to a polyarginine CPP, was tested for its ability to antagonize the pro-apoptotic function of the X-protein. The oligomerization domain peptide was repetitively added at 3, 24, and 48 h to HepG2 cells transiently transfected to express the wild-type and C-terminally-truncated forms of the X-protein. Control transfectants were either left untreated or treated with a peptide containing just the instability domain (aa 140–153), which had been rendered cell-permeable by fusion to a polyarginine CPP. Cells were cultured for a total of 51 h following plasmid transfection, and stained with annexin-V and propidium iodide to detect apoptosis and necrosis, respectively. The majority of transfectants, either left untreated (Fig. 4A) or treated with the control peptide (Fig. 4B) were apoptotic as expected. In contrast, cell apoptosis was markedly reduced by treatment of the cells with the oligomerization domain peptide (Fig. 4A). The oligomerization domain was subsequently shown to be inherently cell-permeable due to the fact that it harbors the CPP Xentry at its N-terminus [21], as illustrated in Fig. 2C; hence modification with polyarginine is probably unnecessary.

4. Discussion

PROTAC-based therapy to target disease-forming proteins is based on exploiting the proteolytic power of the ubiquitin–proteasome system, which rapidly degrades unwanted proteins in cells [2–5]. It is dependent on identifying protein–protein interactive domains in targeted proteins that can serve as target sites to direct the E3 ligase. We hypothesized that the dimerization domain within the N-terminal region of the X-protein could serve as a target site for binding by a PROTAC. Targeting peptides encompassing aa residues 16–30 and 16–35 were defined as the smallest peptides of those tested which showed enhanced binding to the dimerization domain. We further hypothesized that the instability domain of the X-protein could serve as a novel E3 ligase recognition signal (degron). Thus, we designed a novel PROTAC in which the N-terminal dimerization domain of the X-protein was fused to the C-terminal degron domain of the X-protein, essentially bringing the N- and C-terminal ends of the X-protein together, and omitting most of the C-terminal two-thirds of the protein responsible for the majority of X-protein functions.

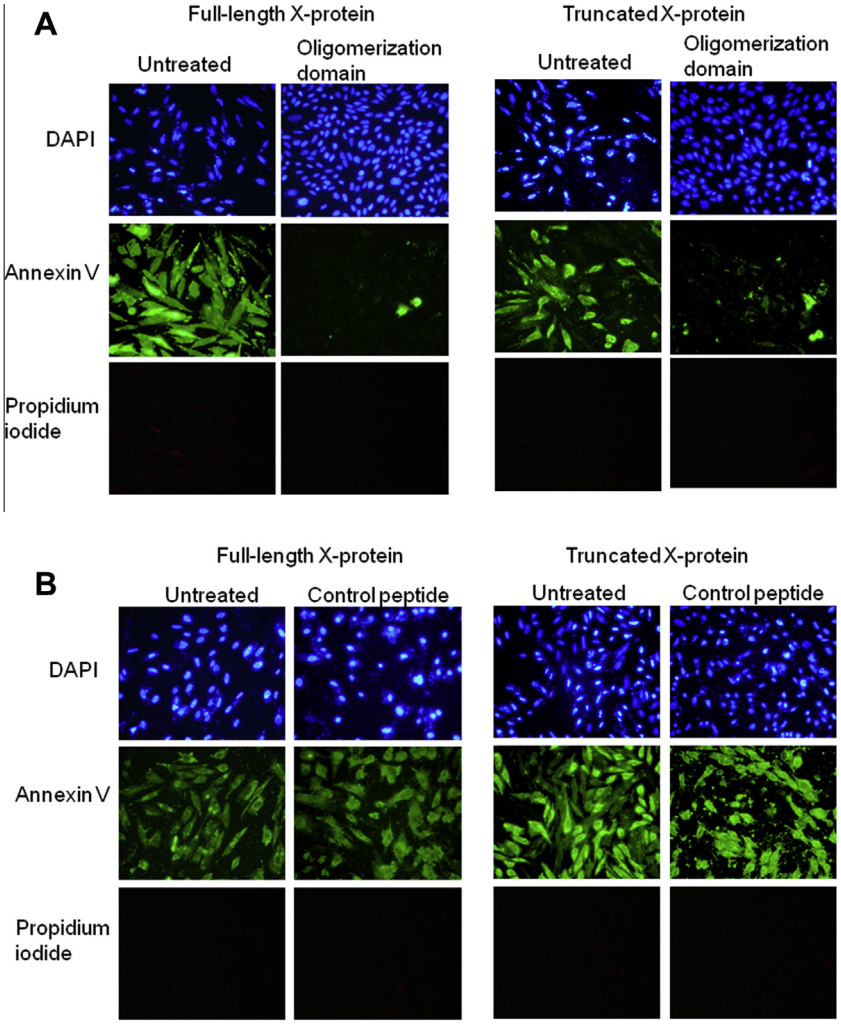

Fig. 4. A stand-alone form of the oligomerization domain antagonizes X-protein-induced apoptosis. (A) The cell-permeable X-protein oligomerization peptide (aa 16–35) was repetitively added at 3, 24, and 48 h to HepG2 cells transiently transfected to express the full-length and C-terminally-truncated forms of the X-protein, as indicated. Control transfectants were left untreated. (B) A control peptide containing just the instability domain (aa 140–153), which had been rendered cell-permeable by fusion to a polyarginine CPP was added to cells as in A. Cells were cultured for a total of 51 h following plasmid transfection, and stained with annexin-V and propidium iodide.

The approach of using CPPs to deliver PROTACs was first described by Rodriguez-Gonzalez, who used polyarginine-modified PROTACS to target androgen and estrogen receptors for the treatment of breast and prostate cancer [7]. Here we also employed a polyarginine sequence to deliver the X-protein-targeting PROTAC to cells. However, we subsequently discovered that the oligomerization domain of the X-protein contains a CPP, designated Xentry [21], hence the PROTAC does not necessarily require modification with polyarginine to render it cell-permeable, though this notion was not tested.

The most commonly employed degron is the ODD domain of hypoxia inducible factor (HIF-1α) which is recognized by the pVHL E3 ligase [6,7]. Here we showed that a degron based on the X-protein instability domain is as equally effective as the ODD domain. The position of the degron within the fusion peptide did not have a marked affect on the activity of the PROTAC. E3 ligase transfers ubiquitin molecules to lysine residues at recognition sites in target proteins [1]. The instability domain of the X-protein variant used in the present study has two lysine residues at aa 139 and 148. Regions of the X-protein that are ubiquinated lie within aa 52–102 and 103–154 [17]. Surprisingly, an X-protein in which all 6 lysines in these regions were mutated was still susceptible to proteasomal degradation, suggesting that the X-protein and potentially the PROTAC described here are subject to both ubiquitin-dependent and -independent proteasomal degradation [17].

The X-protein is subject to mutation and alternative splicing such that C-terminally truncated variants of the X-protein lacking amino acids 134–154, which includes the instability domain are frequent in patients with HCC [15]. The truncated variants are less proapoptotic than wild-type X-protein and promote stronger growth of liver cells [22]. The PROTAC was equally efficient at destroying the wild-type and C-terminally truncated form of the X-protein lacking the instability domain.

Rather than simply binding the target protein, the oligomerization domain of the PROTAC designed here also disrupts X-protein function via acting as a dominant-negative inhibitor capable of inhibiting X-protein-induced apoptosis. Identification of the mechanisms by which the oligomerization domain inhibits X-protein-induced apoptosis is beyond the scope of this report. The most obvious mechanisms include an ability to disrupt the dimerization of the X-protein, or its interaction with effectors. The majority of X-protein functions including enhancement of HBV transcription and replication, and apoptosis are mediated by the C-terminal two-thirds of the X-protein (aa 51–154) [15,18,23,24]. The oligomerization domain interacts with itself and the full-length X-protein, but does not physically interact with

the C-terminal two-thirds of the X-protein [18]. The N-terminal third of the X-protein containing the oligomerization domain antagonizes functions mediated by the C-terminal domain, including repression of the transactivation of HBV and apoptosis [18]. In accord with the present study, retroviral expression of peptide aa 1–78 reduced apoptosis of hepatocyte cell lines mediated by both wild-type X-protein and the aa 1–140 truncated form [15]. In contrast, the peptide did not inhibit pathway activation and apoptosis mediated by the X-protein peptide aa 51–140, clearly demonstrating that peptide aa 1–78 binds directly to the X-protein to exert its inhibitory effects [15]. Retroviral expression of peptide aa 1–78 reduced cell apoptosis by approximately 50% [15], whereas inhibition by the stand-alone oligomerization domain in the current study was near complete. Additional pathways of inhibition of X-protein function by the oligomerization domain may be involved. The X-protein interacts with an array of proteins involved in inhibiting cell apoptosis, any of which might be targets for dominant-negative inhibition by the oligomerization domain. They include Bcl-2-associated X-protein [25], cellular FLICE inhibitory protein [26], the voltage-dependent anion channel [27], hepatitis B X-interacting protein (HBXIP)–survivin complexes [28], and p53 [29].

In summary, cell-penetrating PROTACs based on the oligomerization and instability domains of the X-protein are capable of antagonizing the X-protein and causing its destruction. They warrant testing as therapeutics in the treatment of HBV infection, and/or prevention of HCC.

Acknowledgments

We are grateful for the financial support of the Auckland Medical Research Foundation, New Zealand; Auckland Uniservices Ltd., New Zealand; the Foundation for Research, Science and Technology, New Zealand; and the Maurice and Phyllis Paykel Trust, New Zealand. K.M. was the recipient of a University of Auckland Doctoral Scholarship.

References

[1] S.H. Lecker, A.L. Goldberg, W.E. Mitch, Protein degradation by the ubiquitin–proteasome pathway in normal and disease states, J. Am. Soc. Nephrol. 17 (2006) 1807–1819.
[2] P. Zhou, R. Bogacki, L. McReynolds, P.M. Howley, Harnessing the ubiquitination machinery to target the degradation of specific cellular proteins, Mol. Cell 6 (2000) 751–756.
[3] K.M. Sakamoto, K.B. Kim, A. Kumagai, F. Mercurio, C.M. Crews, R.J. Deshaies, Protacs: chimeric molecules that target proteins to the Skp1–Cullin–F box complex for ubiquitination and degradation, Proc. Natl. Acad. Sci. USA 98 (2001) 8554–8559.
[4] P. Zhou, Targeted protein degradation, Curr. Opin. Chem. Biol. 9 (2005) 51–55.
[5] E.R. Jang, W. Lee, K.B. Kim, Targeted degradation of proteins by PROTACs, Curr. Protoc. Chem. Biol. 2 (2010) 71–87.
[6] J.W. Lee, S.H. Bae, J.W. Jeong, S.H. Kim, K.W. Kim, Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions, Exp. Mol. Med. 36 (2004) 1–12.
[7] A. Rodriguez-Gonzalez, K. Cyrus, M. Salcius, K. Kim, C.M. Crews, R.J. Deshaies, K.M. Sakamoto, Targeting steroid hormone receptors for ubiquitination and degradation in breast and prostate cancer, Oncogene 27 (2008) 7201–7211.
[8] K.M. Sakamoto, Protacs for treatment of cancer, Pediatr. Res. 67 (2010) 505–508.
[9] A.J. Zuckerman, More than third of world's population has been infected with hepatitis B virus, Br. Med. J. 318 (1999) 1213.
[10] S. Murakami, Hepatitis B virus X protein: structure, function and biology, Intervirology 42 (1999) 81–99.
[11] M.A. Feitelson, L.X. Duan, Hepatitis B virus X antigen in the pathogenesis of chronic infections and the development of hepatocellular carcinoma, Am. J. Pathol. 150 (1997) 1141–1157.
[12] M. Motavaf, S. Safari, M. Saffari Jourshari, S.M. Alavian, Hepatitis B virus-induced hepatocellular carcinoma: the role of the virus x protein, Acta Virol. 57 (2013) 389–396.
[13] M.C. Kew, Hepatitis B virus x protein in the pathogenesis of hepatitis B virus-induced hepatocellular carcinoma, J. Gastroenterol. Hepatol. 26 (Suppl. 1) (2011) 144–152.
[14] K.A. McGlynn, W.T. London, The global epidemiology of hepatocellular carcinoma: present and future, Clin. Liver Dis. 15 (2011) 223–243.
[15] H. Li, C.Y. Chi, S. Lee, O.M. Andrisani, The mitogenic function of hepatitis B virus X protein resides within amino acids 51 to 140 and is modulated by N- and C-terminal regulatory regions, J. Virol. 80 (2006) 10554–10564.
[16] Z. Hu, Z. Zhang, E. Doo, O. Coux, A.L. Goldberg, T.J. Liang, Hepatitis B virus X protein is both a substrate and a potential inhibitor of the proteasome complex, J. Virol. 73 (1999) 7231–7240.
[17] J.H. Kim, S.Y. Sohn, T.S.Benedict Yen, B.Y. Ahn, Ubiquitin-dependent and -independent proteasomal degradation of hepatitis B virus X protein, Biochem. Biophys. Res. Commun. 366 (2008) 1036–1042.
[18] S. Murakami, J.H. Cheong, S. Kaneko, Human hepatitis virus X gene encodes a regulatory domain that represses transactivation of X protein, J. Biol. Chem. 269 (1994) 15118–15123.
[19] Y. Guo, J. Hou, Establishment of the consensus sequence of hepatitis B virus prevailing in the mainland of China, Chin. J. Microbiol. Immunol. 19 (1999) 189–2000.
[20] M.H. Lin, S.C. Lo, Dimerization of hepatitis B viral X protein synthesized in a cell-free system, Biochem. Biophys. Res. Commun. 164 (1989) 14–21.
[21] K. Montrose, Y. Yang, X. Sun, S. Wiles, G.W. Krissansen, Xentry, a new class of cell-penetrating peptide uniquely equipped for delivery of drugs, Sci. Rep. 3 (2013) 1661.
[22] H. Zhang, C.L. Shan, N. Li, X. Zhang, X.Z. Zhang, F.Q. Xu, S. Zhang, L.Y. Qiu, L.H. Ye, X.D. Zhang, Identification of a natural mutant of HBV X protein truncated 27 amino acids at the COOH terminal and its effect on liver cell proliferation, Acta Pharmacol. Sin. 29 (2008) 473–480.
[23] H. Tang, L. Delgermaa, F. Huang, N. Oishi, L. Liu, F. He, L. Zhao, S. Murakami, The transcriptional transactivation function of HBx protein is important for its augmentation role in hepatitis B virus replication, J. Virol. 79 (2005) 5548–5556.
[24] H. Tu, C. Bonura, C. Giannini, H. Mouly, P. Soussan, M. Kew, P. Paterlini-Bréchot, C. Bréchot, D. Kremsdorf, Biological impact of natural COOH-terminal deletions of hepatitis B virus X protein in hepatocellular carcinoma tissues, Cancer Res. 61 (2001) 7803–7810.
[25] H.J. Kim, S.Y. Kim, J. Kim, H. Lee, M. Choi, J.K. Kim, J.K. Ahn, Hepatitis B virus X protein induces apoptosis by enhancing translocation of Bax to mitochondria, IUBMB Life 60 (2008) 473–480.
[26] K.H. Kim, B.L. Seong, Pro-apoptotic function of HBV X protein is mediated by interaction with c-FLIP and enhancement of death-inducing signal, EMBO J. 22 (2003) 2104–2116.
[27] Y. Shirakata, K. Koike, Hepatitis B virus X protein induces cell death by causing loss of mitochondrial membrane potential, J. Biol. Chem. 278 (2003) 22071–22208.
[28] H. Marusawa, S. Matsuzawa, K. Welsh, H. Zou, R. Armstrong, I. Tamm, J.C. Reed, HBXIP functions as a cofactor of survivin in apoptosis suppression, EMBO J. 22 (2003) 2729–2740.
[29] L.W. Elmore, A.R. Hancock, S.F. Chang, X.W. Wang, S. Chang, C.P. Callahan, D.A. Geller, H. Will, C.C. Harris, Hepatitis B virus X protein and p53 tumor suppressor interactions in the modulation of apoptosis, Proc. Natl. Acad. Sci. USA 94 (1997) 14707–14712.