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ORIGINAL ARTICLE

Targeting steroid hormone receptors for ubiquitination and degradation in breast and prostate cancer

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Proteolysis targeting chimeric molecules (Protacs) target proteins for destruction by exploiting the ubiquitin-dependent proteolytic system of eukaryotic cells. We designed two Protacs that contain the peptide 'degron' from hypoxia-inducible factor-1α, which binds to the Von –Hippel–Lindau (VHL) E3 ubiquitin ligase complex, linked to either dihydroxytestosterone that targets the androgen receptor (AR; Protac-A), or linked to estradiol (E2) that targets the estrogen receptor-α (ERα; Protac-B). We hypothesized that these Protacs would recruit hormone receptors to the VHL E3 ligase complex, resulting in the degradation of receptors, and decreased proliferation of hormone-dependent cell lines. Treatment of estrogen-dependent breast cancer cells with Protac-B induced the degradation of ERα in a proteasome-dependent manner. Protac-B inhibited the proliferation of ERα-dependent breast cancer cells by inducing G_1 arrest, inhibition of retinoblastoma phosphorylation and decreasing expression of cyclin D1, progesterone receptors A and B. Protac-B treatment did not affect the proliferation of estrogen-independent breast cancer cells that lacked ERα expression. Similarly, Protac-A treatment of androgen-dependent prostate cancer cells induced G_1 arrest but did not affect cells that do not express AR. Our results suggest that Protacs specifically inhibit the proliferation of hormone-dependent breast and prostate cancer cells through degradation of the ERα and AR, respectively.
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Introduction

Prostate cancer is the second most common type of cancer diagnosed in men (Culig *et al.*, 2003; Parkin *et al.*, 2005). Breast cancer is the most common cancer diagnosed in women, which represent 14% of all female cancer deaths (Parkin *et al.*, 2005). The pathogenesis of prostate and breast cancer has been clearly linked to hormone stimulation of the androgen receptor (AR; Huggins, 1967) and the estrogen receptor-α (ERα), respectively (Ellis *et al.*, 2001; Buzdar, 2003). Hormonal and systemic chemotherapy are used for metastatic breast and prostate cancer (Ellis *et al.*, 2001; Buzdar, 2003). Although 66% of breast tumors express ERα and 70% of these respond to hormone therapy, metastatic disease has a very low cure rate and eventually leads to hormone refractory disease despite expression of ERα in 30% of refractory tumors (Clarke *et al.*, 1996). In prostate cancer, the majority of patients (85%) will have an initial favorable response to hormone therapy, however eventually hormone refractory disease occurs approximately 18–24 months after the start of therapy, and most patients succumb to their disease (Santen, 1992; Savarese *et al.*, 2001). Hormone-resistant disease is acquired in over 50% of the cases through molecular and cellular changes that alter the normal activation of the AR and allow prostate cancer cells to grow despite physiologically low serum testosterone levels (Culig *et al.*, 1994; Gaddipati *et al.*, 1994; Bubendorf *et al.*, 1999; Craft *et al.*, 1999; Taplin *et al.*, 1999; Godoy-Tundidor *et al.*, 2002; Ueda *et al.*, 2002; Bakin *et al.*, 2003; Franco *et al.*, 2003). Mechanisms of hormone resistance include overexpression, mutation, or indirect activation of AR. Interestingly, the AR continues to be expressed and is required for cell growth and survival (Linja *et al.*, 2001; Eder *et al.*, 2002; Zegarra-Moro *et al.*, 2002; Liao *et al.*, 2005). Presently, there is no effective treatment for androgen refractory disease (Thiele *et al.*, 1999; Moul, 2000).

Considering the importance of the expression and activity of hormone receptors in breast and prostate

cancer, we designed a new approach to treatment. This technology, which is based on heterobifunctional small molecules known as proteolysis targeting chimeric molecules (Protacs), exploits the unique characteristics of the ubiquitin proteasome system in eukaryotic cells. Protacs are bridging molecules that recruit a ubiquitin ligase (E3) to the target of interest. These heterobifunctional small molecules contain two moieties; the first moiety (degron) binds to a ubiquitin ligase, whereas the second moiety is a ligand for the target that will be degraded. Consequently, when these moieties are joined, they link the target to a ubiquitin ligase, resulting in ubiquitination and degradation of the target (Figure 1a). In prior work, we established 'proof of principle' that Protacs are effective *in vitro* to recruit proteins for ubiquitination and degradation (Sakamoto *et al*., 2001). We also showed that Protacs based on the IκBα peptide can induce the ubiquitination of purified ERα and degradation of GFP-AR expressed in 293 cells, but only when these Protacs were microinjected into cells (Sakamoto *et al*., 2001, 2003). To develop Protacs for *in vivo* application, a cell permeable version of Protac was synthesized (Schneekloth *et al*., 2004). The previously used degron (IκBα) required phosphorylation on two serine residues to be recognized by SCFβ−TRCP (Karin and Ben-Neriah, 2000), which made the resulting Protacs relatively impermeable and susceptible to phosphatases. To avoid this problem, the IKBα peptide was replaced by a hydroxyproline-containing peptide from hypoxia-inducible factor-1α (HIF-1α), which is recognized by the Von –Hippel–Lindau (VHL) ubiquitin ligase complex and is not dependent on phosphorylation (Kim and Kaelin, 2004; Schneekloth *et al*., 2004; Bargagna-Mohan *et al*., 2005). These Protacs contain a pentapeptide degron from HIF-1α (Bargagna-Mohan *et al*., 2005), linked to dihydroxytestosterone (DHT) to target the AR (Protac-A), or linked to estradiol (E2) to target the ERα (Protac-B; Figure 1b). We hypothesized that these Protacs should be cell permeable and induce degradation of AR or ERα, respectively. In this paper, we report that these two Protacs lead to ubiquitination and degradation of endogenous AR and ERα. The reduction of receptor protein levels occurs through a proteasome-dependent pathway, and induces cell cycle and growth arrest in hormone-dependent but not hormone-independent tumor cell lines.

Results

Specific effect of Protac in hormone-dependent cell lines

We used the lymph node carcinoma of prostate (LNCaP) cell line as the androgen-dependent prostate

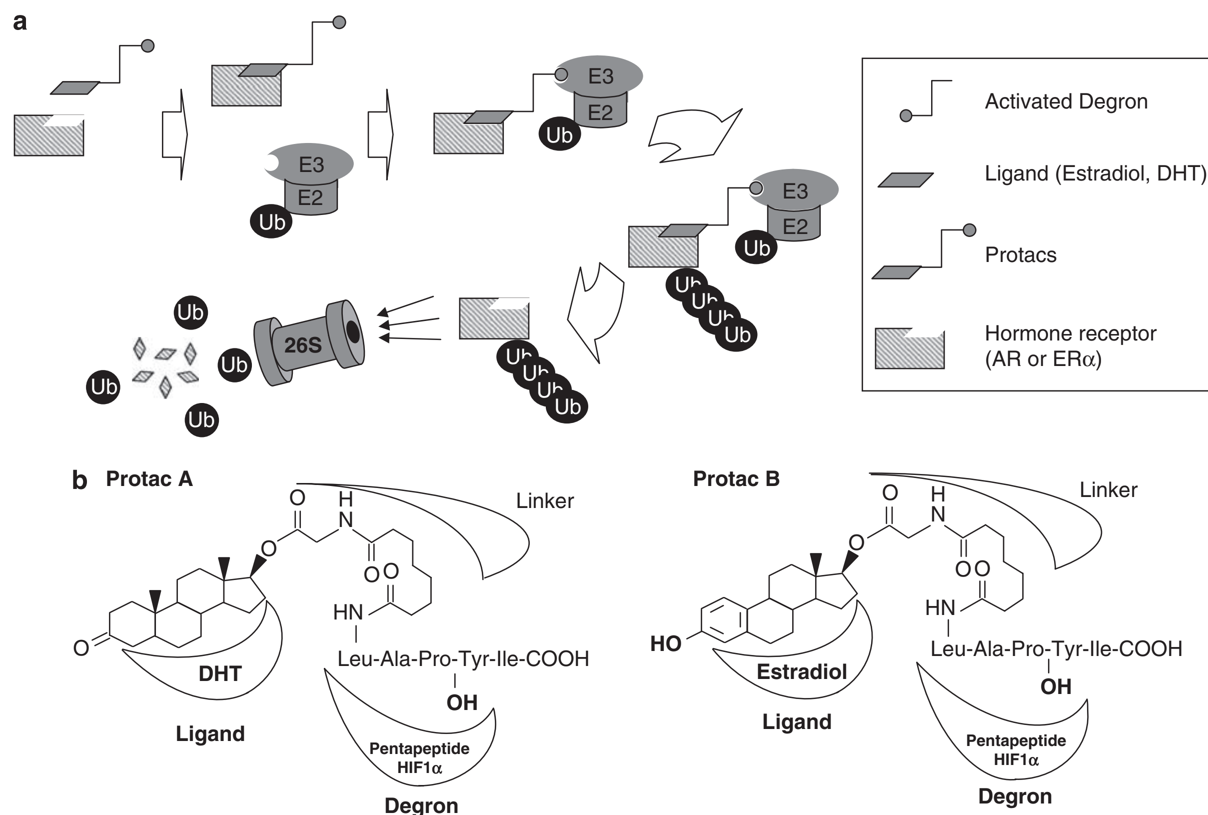


Figure 1 Proteolysis targeting chimeric molecule (Protac) model. (**a**) Two moieties form the chimeric compound (Protac). The first moiety is the activated degron, hydroxylated pentapeptide of hypoxia-inducible factor-1α (HIF-1α) that would recruit the activity of Von –Hippel–Lindau ubiquitin ligase (E3). The second moiety is the natural ligand of the target that we wish to degrade, Estradiol for estrogen receptor-α or dihydroxytestosterone for androgen receptor. This heterobifunctional small molecule is a specific bridging molecule for protein degradation. Ub stands for ubiquitin. (**b**) Protacs-A and B. The specificity is because of the ligand that interacts with the target. The ubiquitin ligase activity is recruited by the hydroxypentapeptide of HIF 1α.

cancer model. LNCaP cells express the AR and can only proliferate in medium that contains androgens (Supplementary Figure S1A). As a negative control, we used PC-3 and DU-145 cells, which are both androgen independent and do not express AR (Supplementary Figure S1A). For the breast cancer model, we used MCF-7 and T47D as hormone-dependent cell lines, and SKBr3 as the hormone-independent cell line (Supplementary Figure S1B). SKBr3 does not express ERα. As MCF-7, T47D and LNCaP cells are hormone dependent, we hypothesized that treatment with Protac would affect the growth of these cells. In both breast and prostate cancer models, the proliferation of hormone-dependent cell lines was inhibited by Protac treatment. Protac-A (DHT-Protac) affected the proliferation of LNCaP cells (IC_{50}(72 h) = 12.5 μM, IC_{50}(144 h) = 1.52 μM) whereas androgen-independent PC-3 (Figure 2a) or DU-145 cells (data not shown) were not affected at the same concentrations, which suggests that Protac-A is specific for its target. We also investigated the effect of DHT on the proliferation of LNCaP cells (Figure 2a), when used at the IC_{50} of Protac-A (12.5 μM), DHT had no effect on LNCaP cell proliferation. Therefore, Protac-A requires the peptidic moiety (HIF1α pentapeptide degron) to inhibit LNCaP cell proliferation. The same specificity was observed in breast cancer cells, where the proliferation of estrogen-dependent cell lines (MCF-7 and T47D) was inhibited by Protac-B treatment (IC_{50}(72 h) = 50 μM and IC_{50}(72 h) = 16 μM, respectively), but there was no effect on proliferation by treatment with estradiol (Figure 2b). Meanwhile, proliferation of the estrogen-independent cell line SKBr3 was not affected by Protac-B (Figure 2b). The reduction in proliferation of hormone-dependent (but not hormone-independent) cell lines treated with Protacs is presumably because of downregulation of hormone receptor. To test this hypothesis, we examined by western blot analysis the

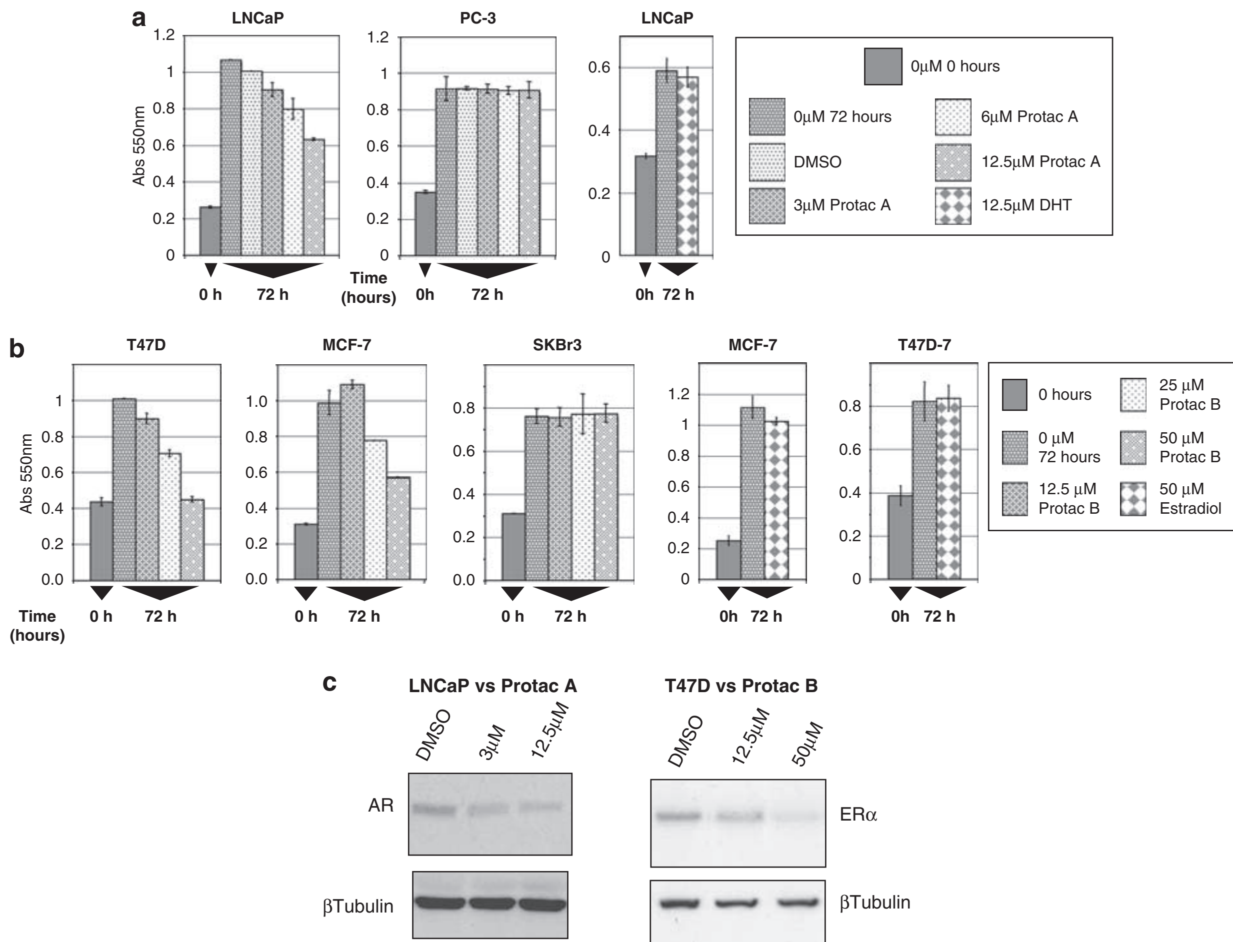


Figure 2 Specific effect of proteolysis targeting chimeric molecule (Protac) treatment in prostate and breast cancer cell lines. (**a**) Effects of Protac-A treatment for 72 h on proliferation of prostate cancer cell lines. IC_{50} = 12.5 μM for LNCaP cells, whereas PC-3 cells were not affected at the same concentrations. (**b**) Effects of Protac-B on proliferation of breast cancer cell lines. MCF-7 and T47D had an IC_{50} = 50 μM and IC_{50} = 16 μM, respectively, whereas SKBr-3 was not affected at the same concentrations. Neither dihydroxytestosterone nor estradiol affected the proliferation of cells at the same concentrations used for Protacs. (**c**) Effects of Protacs on receptor protein levels. LNCaP or T47D cells were treated for 72 h with increasing concentrations of Protac-A or -B, respectively. The proliferation in all cases was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method. Absorbance at 550 nm corresponds to living cells. The graphs show the mean values of a single experiment performed in triplicate, representative of three independent experiments.

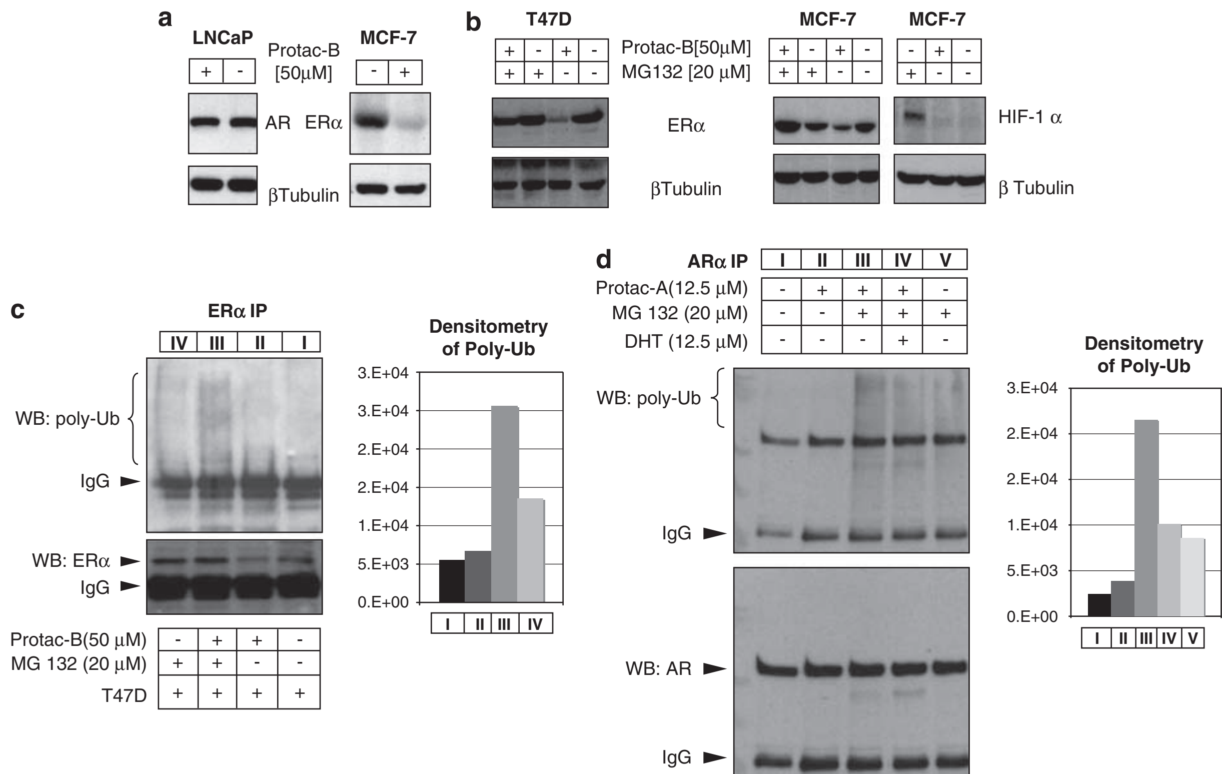


Figure 3 Proteolysis targeting chimeric molecules (Protacs) induce ubiquitination and proteasome-dependent degradation. (**a**) Western blot analysis with androgen receptor (AR), estrogen receptor-α (ERα), β-tubulin antisera after treatment with Protac-B. LNCaP and MCF-7 were treated with 50 μM of Protac-B for 24 h. (**b**) Western blot analysis with anti-ERα, β-tubulin and hypoxia-inducible factor-1α antisera and lysates from MCF-7 and T47D cells. Cells were treated with MG132, and Protac-B for 6 h at the indicated concentrations. (**c**) Immunoprecipitation with anti-ERα antisera and immunoblot with anti-ERα and ubiquitin antisera. T47D were treated for 6 h with Protac-B and MG132 at the indicated concentrations. The graph corresponds to densitometry of the shown ubiquitinated ERα western blot. (**d**) Immunoprecipitation with anti-AR antisera and immunoblot with anti-AR and ubiquitin antisera. LNCaP cells were treated for 6 h with Protac-A and MG132 at the indicated concentrations. The graph corresponds to densitometry of the shown ubiquitinated AR western blot. These experiments were repeated three times.

levels of hormone receptor in cells treated with Protac. We observed a significant decrease in the levels of AR and ERα at Protac concentrations that inhibited proliferation (Figure 2c).

Protacs induce ubiquitination and proteasome-dependent degradation of the targeted hormone receptors

One of the advantages of Protacs is their target specificity. The specificity is conferred by the target-binding ligand, DHT in the case of Protac-A (targeting AR) and 17β-estradiol in Protac-B (targeting ERα; Figure 1b). As an example of this specificity, AR protein levels were not affected by treatment of LNCaP cells with the ERα-specific Protac-B (Figure 3a). We next investigated the mechanism by which Protacs brought about decreases in AR and ERα levels. In both MCF-7 and T47D cells, the proteasome inhibitor MG132 blocked downregulation of ERα by Protac-B (Figure 3b). These results suggest that Protac-B activates degradation of ERα by the proteasome.

To determine whether Protac-dependent turnover of ERα and AR involves polyubiquitination, we immunoprecipitated ERα from breast and AR from prostate cancer cells, respectively, following treatment with Protac and MG132 (Figures 3c and d). The immunoprecipitates were subsequently blotted with anti-ubiquitin to assess the levels of ubiquitin-conjugated ERα. ERα ubiquitination increased in the presence of Protac-B and proteasome inhibitor (MG132) in comparison to treatment with proteasome inhibitor alone (Figure 3c). This suggests that ERα degradation in treated cells is because of a Protac-dependent increase in receptor ubiquitination. Similarly, enhanced ubiquitination of AR ubiquitination was observed in prostate cancer cells treated with Protac-A (Figure 3d).

Our prior data indicated that treatment with Protacs based on the HIF1α resulted in degradation of their targets through association with the VHL E3 ligase. To address whether recruitment of VHL activity to ERα by Protac-B competes with the ubiquitination and degradation of endogenous HIF1α, we examined HIFα levels in cells treated with this Protac. HIFα levels did not increase in cells treated with Protac-B but did increase in cells treated with proteasome inhibitor (Figure 3b). Thus, the effect of Protac on the proliferation of hormone-dependent cell lines correlates with downregulation of the targeted receptor and does not appear to depend on accumulation of the VHL substrate HIF1α.

The induction of targeted receptor degradation by Protac treatment inhibits receptor signaling, leading to cell cycle arrest

To understand the mechanism by which Protac inhibits breast and prostate cancer cell proliferation, we investigated the expression of known target genes activated by ERα, cyclin D1 and progesterone receptor (PR) (Klein-Hitpass *et al*., 1988; Savouret *et al*., 1991; Butt *et al*., 2005). Treatment of estrogen-dependent cells with Protac-B resulted in decreased expression of cyclin D1 and PR concomitant with decreased ERα levels (Figure 4a). Consistent with the idea that Protac-induced turnover of hormone receptors silences downstream signaling pathways that drive tumor cell proliferation, treatment of breast and prostate cancer cells with Protacs resulted in dephosphorylation of the retinoblastoma (Rb) protein (Figures 4a and b) and cell cycle arrest in G_1 (Figure 4b). Rb is a critical regulator of the G_1/S transition and its activation by dephosphorylation leads to a cell cycle arrest in G_1. We also observed a dose-dependent dephosphorylation of Rb at 72 h in Protac-treated LNCaP and MCF-7 cells (data not shown). To address the specificity of the molecular responses to Protacs, we tested whether they can be reversed by the addition of competitor steroid hormone. We treated MCF-7 and T47D cells with 50 μM of Protac-B and 50 μM of estradiol (Figure 4a). In both cases, we observed that treatment of cells with estradiol and Protac resulted in minimal or no downregulation of cyclin D1, PR A and B, and phospho-Rb. Similarly, we could block effects of Protac-A treatment on Rb phosphorylation in prostate cancer LNCaP cells by simultaneously treating with a 10-fold excess of DHT (Figure 4b).

Although simultaneous treatment with Protac and hormone reversed the inhibitory effect of Protacs on ERα and AR function, the addition of hormone did not block downregulation of the hormone receptors. This is likely because of the fact that steroid hormones induce turnover of their receptors. In particular, the estradiol-induced degradation of ERα has been studied in detail (Wijayaratne and McDonnell, 2001). Estradiol-induced turnover of ERα is intimately linked to ERα function and thus is tightly correlated with ERα activity. On the

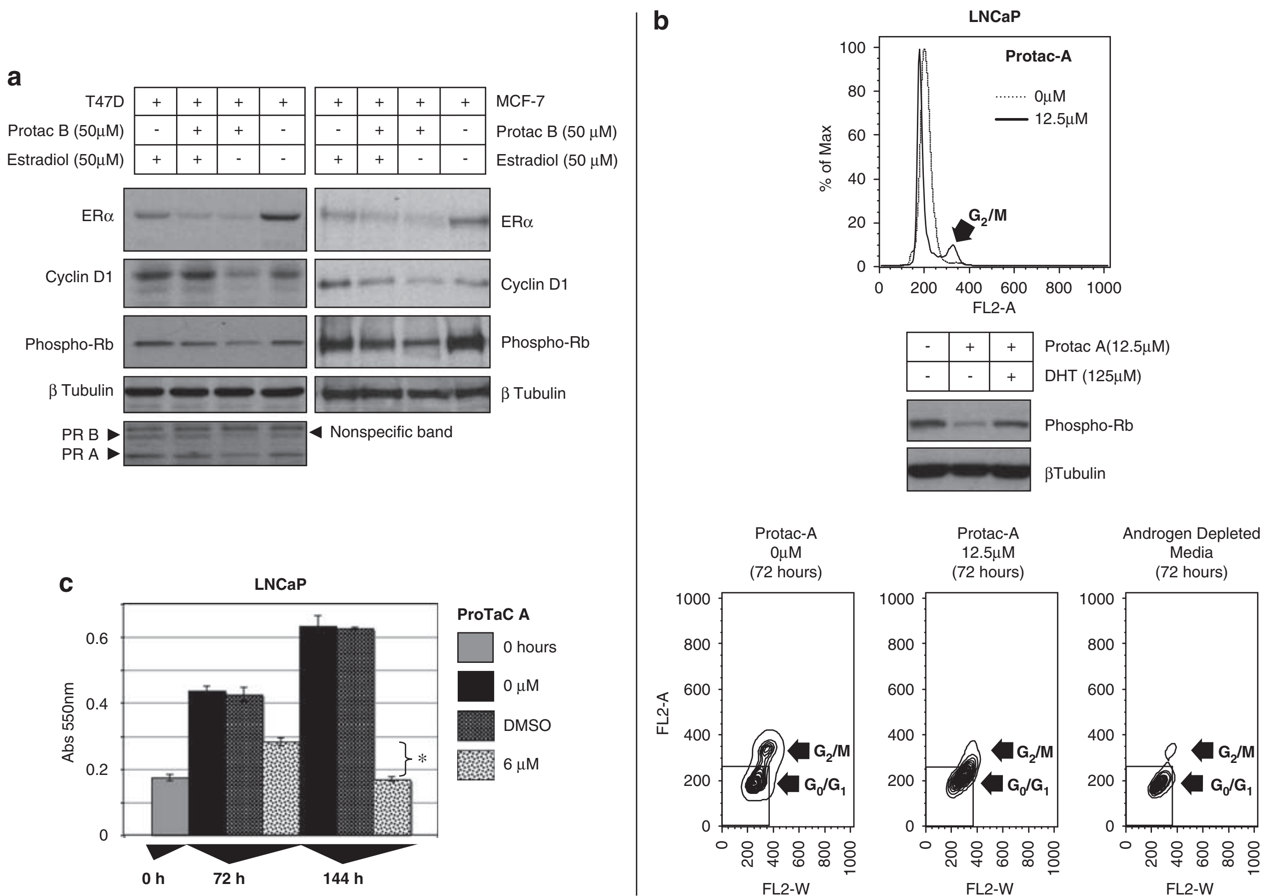


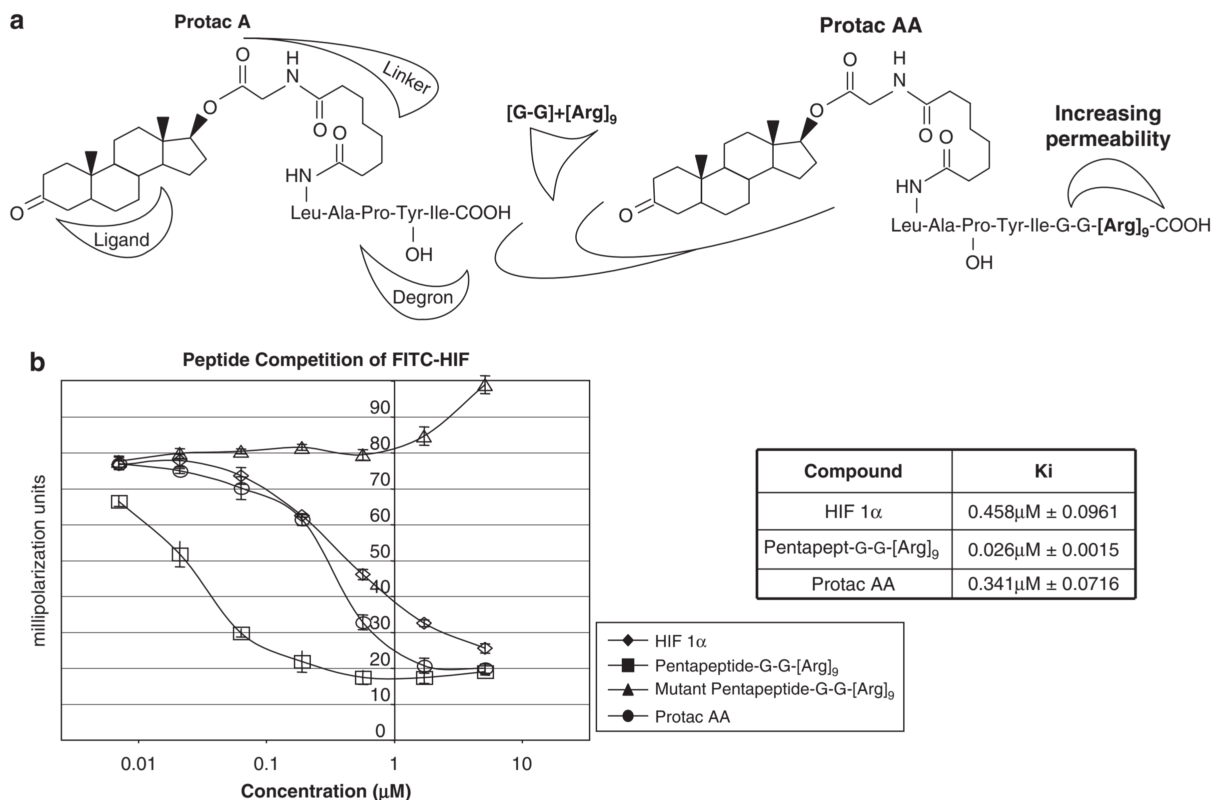
Figure 4 Proteolysis targeting chimeric molecule (Protac) treatment induces cell cycle arrest in hormone-dependent cell lines. (**a**) Expression of protein levels of phosphorylated retinoblastoma (Rb), β-tubulin, progesterone receptor, cyclin D1 and estrogen receptor-α after treating T47D and MCF-7 cells with Protac-B for 6 h. (**b**) Comparison of cell cycle profile corresponding to LNCaP cells treated with Protac-A for 72 h. Cells were stained with propidium iodide. Expression of protein levels of phosphorylated Rb and β-tubulin in LNCaP cells after being treated with Protac-A during 6 h. (**c**) LNCaP cell growth after 6 days of treatment with Protac-A. (}*) indicates the decrease in cell viability during the past 72 h of treatment. The proliferation in all cases was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method. Absorbance at 550 nm corresponds to living cells. The graph shows mean values of a single experiment performed in triplicate, representative at least of three independent experiments with similar results.

other hand, ectopic degradation of hormone receptors induced by Protacs is unlikely to be linked to receptor-dependent transcription, and thus is not expected to correlate with ERα function.

To determine the durability of the antagonistic effect of Protacs, we investigated the effects of prolonged Protac treatment on prostate and breast cancer cells. After 6 days of continuous Protac treatment, proliferation of LNCaP cells was still inhibited and there were fewer viable cells than after 3 days of treatment (Figure 4c). These data suggest that prolonged treatment with Protac may be cytotoxic and induce apoptosis. The same extended treatment did not affect the proliferation of androgen-independent PC-3 cells (data not shown). This result is in agreement with observations reported by other groups that AR is important for the proliferation and survival of hormone-dependent prostate cancer cells (Linja *et al*., 2001; Eder *et al*., 2002; Zegarra-Moro *et al*., 2002; Liao *et al*., 2005). Previously published work demonstrated that siRNA against AR resulted in apoptosis, however, only after 4 days. Therefore, prolonged treatment of Protac may have additional advantages of activating apoptosis in prostate and breast cancer cells.

Rationale for the design of new Protacs

Several possibilities can be envisioned to improve the activity and specificity of Protacs. Targeting new proteins would require a change in the ligand moiety. Protac activity can also be modulated by altering membrane permeability or the affinity for the E3 ligase. Reports have demonstrated that addition of a poly-arginine sequence to peptides enhances cellular entry (Kirschberg *et al*., 2003). To increase cell permeability of Protac, a polyarginine tail and two glycine residues were added (Figure 5a; Kirschberg *et al*., 2003). To evaluate interaction of polyarginine-containing ligand with VHL, we employed a fluorescence polarization assay to measure the ability of the new peptide to compete out binding of a fluorescently labeled HIF-1α degron peptide (Asp-Glu-Ala-Leu-Ala-Pro(OH)-Tyr-Ile-Pro-Asp) to VHL (Figure 5b). The addition of a polyarginine tail did not compromise the affinity of pentapeptide for VHL (pentapeptide-G-G-(arg)$_9$: Leu-Ala-Pro(OH)-Tyr-Ile-Gly-Gly-(D-Arg)$_9$, $K_i = 0.026\,\mu M$; Figure 5b); in fact, affinity was enhanced by ~17-fold compared to the natural hydroxyproline-containing pentapeptide. As a negative control, we used a mutant pentapeptide (mutant pentapeptide-G-G-(Arg)$_9$:



Compound	Ki
HIF 1α	0.458μM ± 0.0961
Pentapept-G-G-[Arg]$_9$	0.026μM ± 0.0015
Protac AA	0.341μM ± 0.0716

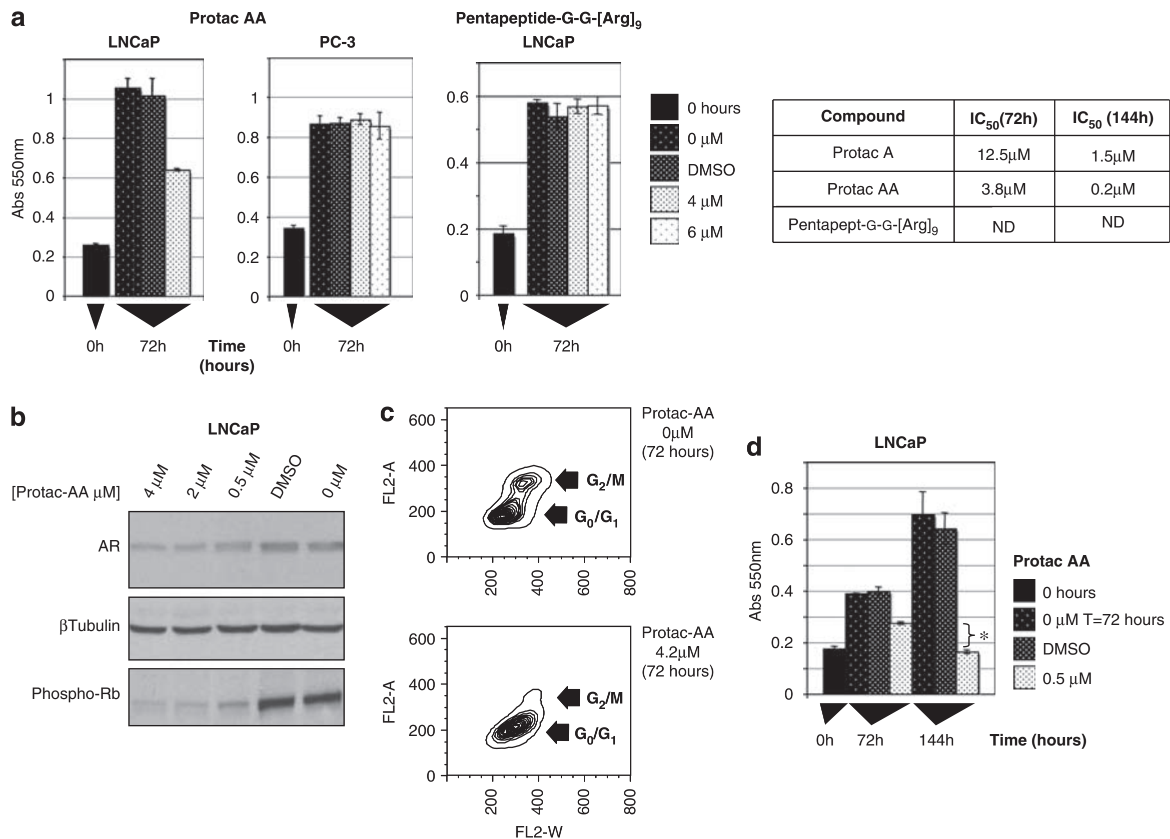
Figure 5 Modification of proteolysis targeting chimeric molecule (Protac) to target androgen receptor. (**a**) Scheme of molecular structure of Protacs-A turning into Protacs-AA after the addition of a -G-G-[Arg]$_9$ (polyarginine tail) to the pentapeptide. (**b**) Competitive binding assay with Von –Hippel–Lindau (VHL). Fluorescent hypoxia-inducible factor-1α (HIF-1α; Asp-Glu-Ala-Leu-Ala-Pro(OH)-Tyr-Ile-Pro-Asp-FITC) is the control competitor for the union with VHL ubiquitin ligase. HIF-1α: (Asp-Glu-Ala-Leu-Ala-Pro(OH)-Tyr-Ile-Pro-Asp). Pentapeptide-Gly-Gly-[D-Arg]$_9$: Leu-Ala-Pro(OH)-Tyr-Ile-Gly-Gly-[D-Arg]$_9$. Mutated pentapeptide-Gly-Gly-[Arg]$_9$: Leu-Ala-Ala-Tyr-Ile-Gly-Gly-[D-Arg]$_9$. Represented in the graphs are mean values of a single experiment performed in triplicate, representative of three independent experiments with similar results. Standard deviations were less than 10% of the total value in each point. Ki's were estimated by interpolation and represent the Ki's of the shown experiment.

Leu-Ala-Ala-Tyr-Ile-Gly-Gly-(D-Arg)$_9$) lacking the critical hydroxyproline ligand. The mutant peptide did not detectably bind VHL (Figure 5b). Finally, we measured the affinity of the new Protac (Protac-AA) for VHL (Figure 5b). This new molecule consists of DHT as the ligand and the peptide (pentapeptide-G-G-(Arg)$_9$: Leu-Ala-Pro(OH)-Tyr-Ile-Gly-Gly-(D-Arg)$_9$) as the degron that is recognized by VHL (Figure 5a). The affinity of this Protac for VHL was very similar to the control HIF1α peptide with $K_i = 0.341$ and $0.458\,\mu$M, respectively. Thus, we would expect an improvement of the activity of the Protac-AA over the Protac-A because of the expected enhancement in cell permeability.

Protac-AA activity in prostate cancer cell lines

To test the effects of Protac-AA, we treated prostate cancer cell lines LNCaP and PC3 for 72 h (Figure 6). The new peptide increased the activity of Protac three to seven times to an IC_{50} of $3.8\,\mu$M at 72 h and an IC_{50} of $0.216\,\mu$M at 144 h (Figure 6a). Growth of PC-3 cells was not affected by Protac-AA at concentrations that suppressed the growth of LNCaP cells (Figure 6a). The new peptide by itself (that is without the DHT ligand; Leu-Ala-Pro(OH)-Tyr-Ile-Gly-Gly-(D-Arg)$_9$) did not affect proliferation of LNCaP cells (Figure 6a). Thus, the polyarginine tail increased the activity of the Protac without altering its specificity.

To determine the effects of Protac-AA on AR levels, we treated LNCaP cells and examined AR expression. Protac reduced the protein level of AR, which was associated with activation of pRb (Figure 6b). Dephosphorylation of pRb was correlated with cell cycle arrest in G_1 (Figure 6c), which was also observed with Protac-A treatment and with androgen deprivation (Figure 4b; Knudsen *et al*., 1998). This more active Protac also induced a reduction in the number of viable cells after prolonged treatment (Figure 6d), suggesting the possibility of specific toxicity similar to previous reports where AR was downregulated by siRNA for several days (Linja *et al*., 2001; Eder *et al*., 2002; Zegarra-Moro *et al*., 2002; Liao *et al*., 2005). These results demonstrate that a Protac that was modified to enhance its



Compound	IC_{50}(72h)	IC_{50} (144h)
Protac A	12.5µM	1.5µM
Protac AA	3.8µM	0.2µM
Pentapept-G-G-[Arg]$_9$	ND	ND

Figure 6 Proteolysis targeting chimeric molecule (Protac) AA activity in prostate cancer cell lines. (**a**) LNCaP and PC-3 cells were treated for 72 h at increasing concentrations of Protac-AA. LNCaP cell growth was inhibited at an $IC_{50} = 3.8\,\mu$M, whereas PC-3 cells were not affected. LNCaP cells were also treated with increasing concentrations of pentapeptide-G-G-[Arg] with no growth inhibition at the same range of concentrations. (**b**) Expression of androgen receptor, phosphorylated retinoblastoma and β-tubulin protein levels in LNCaP cells treated for 72 h with increasing concentrations of Protac-AA. (**c**) Comparison of cell cycle profile corresponding to treated and untreated LNCaP cells with Protac-AA during 72 h. DNA was stained using propidium iodide. (**d**) Viable cells after 6 days of treatment of LNCaP cells with Protac-AA. (}*) indicates the decrease in cell viability during the past 72 h of treatment. Measurement of viable cells was performed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method. Absorbance at 550 nm corresponds to living cells. The graph shows mean values of a single experiment performed in triplicate, representative at least of three independent experiments with similar findings. IC_{50}'s were estimated by interpolation and represent the mean of four independent experiments performed in triplicate.

permeability in cells maintained high affinity interaction and specificity for its target protein.

Discussion

Inhibiting AR or ERα activity has become one of the major goals in controlling the growth of hormone-dependent prostate or breast tumors, respectively. A main objective of the pharmaceutical and biotechnology industries is to isolate compounds that inhibit proteins like AR and ERα that contribute to disease. A common way of doing this is to develop an *in vitro* or *in vivo* high-throughput screening assay for the protein of interest, and then to screen large libraries of natural or man-made compounds for those that inhibit the targeted function (Broach and Thorner, 1996). A second approach is to employ structure-based drug design to create a molecule that will insinuate itself into the active site of the target (Blundell, 1996; Hogan, 1996; Verdine, 1996). Although these methods represent the current state-of-the-art in drug discovery, they both suffer from serious limitations. First, both are laborious, expensive and frequently fail to identify a good lead compound that inhibits the desire target. Second, by their very nature, high-throughput screens are typically limited to identifying substances that interact with the target in a functionally relevant manner. Given that the active site of an enzyme compromises only a small fraction of its total surface area, many compounds that can bind a target may escape detection in these assays. Third, a corollary to the previous limitation is that both high-throughput screens and structure-based drug design are suitable only for proteins with readily assayable activities or well-defined active sites. To address some of these limitations we have developed Protacs that can be used to downregulate any protein that has a known ligand, regardless of whether the target has assayable activity.

In this work, we employed Protacs to downregulate the ERα and AR in breast and prostate cancer cells, respectively. Our results demonstrated that Protacs can induce chemical knockdown of these oncogenic proteins with specificity dictated by the physiologic ligand of the selected target. Furthermore, chemical knockdown was achievable by Protac-dependent ubiquitination and proteasome-dependent degradation of endogenous AR and ERα. Our data also showed that the decrease in ERα levels interfered with receptor signaling as measured by reduced expression of the target genes cyclin D1 and PR A and B. The effects of Protacs in receptor signaling were inhibited by competition with E2 or DHT demonstrating again the specificity of Protacs to their targets. We observed that, whereas Protac-B treatment induces a dramatic reduction on ERα levels, the addition of E2 in competition with Protac-B seemed to partially decrease the downregulation of receptor levels (Figure 4a). A possible explanation for the lack of a synergistic effect would be that estradiol stimulation induces approximately 50% decrease in ERα levels as it has been shown in previous studies (Pink and Jordan, 1996). After this decrease in receptor levels, a stable steady-state level of receptor expression is established during the continued presence of estradiol, which does not happen with Protacs treatment. These observations would explain why ERα degradation induced by E2 was less than that induced by Protac (Figure 4a). The addition of estradiol to Protac-B treatment could result in a competition between them to bind to the same domain in the receptor thereby inducing two different rates of degradation. The combined treatment of both E2 and Protac-B lead to greater degradation than with E2 treatment alone, but less ERα degradation than with only Protac-B treatment (Figure 4a). Estradiol binding to ERα is known to result in ERα ubiquitination for 26S proteasome degradation (Wijayaratne and McDonnell, 2001; Preisler-Mashek *et al*., 2002). ERα degradation by the proteasome has also been closely linked to ERα transcriptional activation (Lonard *et al*., 2000; Reid *et al*., 2003) but this has been controversial depending on the model system (Alarid *et al*., 2003; Fan *et al*., 2004; Duong *et al*., 2007). Although we observed ubiquitin-dependent degradation of ERα following Protac treatment, this does not imply that estrogen specific genes are transcribed (Figure 4a).

In addition to the proximal effects on receptor signaling, treatment with Protacs also led to dephosphorylation of pRb and cell cycle arrest in G_1. Importantly, Protacs had no effect on the proliferation of hormone-independent breast and prostate cancer cell lines, documenting the specificity of their action.

It is interesting to consider how Protacs compare to other approaches for abrogating hormone receptor signaling activity. The effects of ERα-directed Protac are in agreement with those that described a decrease in cyclin D1 gene expression and pRb phosphorylation, and G_0/G_1 cell arrest in MCF-7 cells treated with the pure antiestrogens ICI 182780 and ICI 164384 (Doisneau-Sixou *et al*., 2003). Like the ERα-directed Protac described here, pure antiestrogens also induces ERα degradation (Dauvois *et al*., 1992; Borras *et al*., 1996; Pink and Jordan, 1996), and the loss of ERα not only abrogates the transcriptional effect of estrogen, but also blocks the activation of ERα by other growth factors (Rau *et al*., 2005). Previous work has demonstrated that knocking down AR by siRNA in hormone-dependent cells resulted in cell cycle arrest over a longer period of time (beyond 72 h) and apoptosis after G_1 arrest (Linja *et al*., 2001; Eder *et al*., 2002; Zegarra-Moro *et al*., 2002; Liao *et al*., 2005). Similar responses were observed upon extended incubation of hormone-dependent prostate cancer cells with AR-targeted Protac. It is notable that many prostate refractory tumors still require AR. AR is overexpressed or mutated in these cells such that it remains functional but its affinity for or response to ligands is altered (Culig *et al*., 1994; Gaddipati *et al*., 1994; Bubendorf *et al*., 1999; Craft *et al*., 1999; Taplin *et al*., 1999; Godoy-Tundidor *et al*., 2002; Ueda *et al*., 2002; Bakin *et al*., 2003; Franco *et al*., 2003). Extended antihormonal therapy of breast and prostate cancer can select for a subpopulation of tumor cells in which the drug acts as an agonist instead of an antagonist—as in

the case of flutamide in prostate cancer or tamoxifen in breast cancer (Rau *et al*., 2005), resulting in progression of disease and death (Culig *et al*., 1999; Taplin *et al*., 1999). Protac technology could potentially be useful in treating these classes of disease (mutated or overexpressed receptor but still with affinity for the ligand) because the inhibitory effect on receptor activity is based on the induction of receptor degradation. In many cases the overexpression of AR and ERα in prostate and breast cancer confers resistance to hormone therapy. Theoretically, in those cases Protac would reduce the receptor levels, as we have shown in tumor cell lines. Our finding that receptor degradation by Protac appears to be independent of the relative levels of receptor expression suggests that reduction in receptor levels might increase the sensitivity of tumor cells to hormone therapy. In those cases that the resistance is because of simple receptor overexpression, the best case scenario is that Protac treatment alone blocks receptor signaling.

Although these results are promising, the concentrations of Protacs required for growth inhibition are too high for *in vivo* studies in preclinical animal models. To address this problem, we modified the Protacs to determine whether their cell permeability could be enhanced without compromising affinity or specificity for the target protein. We added a polyarginine tail that should improve the permeability and increase the solubility of Protacs (Kirschberg *et al*., 2003). The polyarginine-containing Protac bound with good affinity to VHL and induced the downregulation of AR and G_1 arrest, albeit at 3- to 4-fold lower concentrations than the original Protac-A. Future work will focus on the development of multiple Protacs that can potentially target additional proteins that contribute to the growth of cancer cells.

Materials and methods

Cell cultures

All cell lines used in this study were maintained under standard conditions of temperature (37 °C), humidity (95%) and carbon dioxide (5%). The following cell lines were used: LNCaP (American Type Culture Collection (ATCC) number CRL-1740), PC-3 (ATCC number CRL-1435), DU-145 (ATCC number HTB-81), T47D (ATCC number HTB-133), MCF-7 (ATCC number HTB-22) and SKBr-3 (ATCC number HTB-30). They were maintained in phenol-red-free RPMI 1640 (Gibco number 11835) supplemented with 10% of fetal bovine serum (FBS; Omega Scientific number FB-01) or 10% of charcoal dextran treated FBS (ctFBS; Omega Scientific number 800-799-5873). ctFBS signifies serum without steroids and it was used in experiments where we wanted to see the requirements of steroids by the different cell lines. The growth in this media (RPMI 1640, 10% ctFBS) was determined to assess hormone dependence.

Cell proliferation and viability assays

All proliferation assays were based on the (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) method. This was based on measuring living cells through mitochondrial dehydrogenase activity (Sigma Inc., Saint Louis, MO, USA). Cells were plated in a 96-well, 10 000 cells per 100 μl/well. After 24–48 h of being plated, the cells were treated with Protac. New media and Protac were replaced after 72 h of experiment when experiments were longer than 72 h. At the end of the experiment, the media was removed and dimethyl sulfoxide was added as MTT solubilization solution. Absorbance was measured at 550 nm.

Peptides, drugs and compounds

We used different compounds for different treatments. MG 132 is a proteasome inhibitor (Calbiochem Inc., Gibbstown, NJ, USA). β-Estradiol was purchased from Sigma-Aldrich Inc. (Saint Louis, MO, USA). All peptides were purchased from GL Biochem Ltd (Shanghai, China); pentapeptide-G-G-$[Arg]_9$ = (H-Leu-Ala-Pro(OH)-Tyr-Ile-Gly-Gly-[D-Arg]$_9$-OH) and mutant pentapeptide-G-G-$[Arg]_9$ = (H-Leu-Ala-Ala-Tyr-Ile-Gly-Gly-[D-Arg]$_9$-OH). Protac was synthesized by coupling β-estradiol to the pentapeptide and pentapeptide-G-G-$[Arg]_9$ as referenced before (Sakamoto *et al*., 2003; Bargagna-Mohan *et al*., 2005).

Western blot analysis

Cells were lysed in radio immunoprecipitation assay (RIPA) buffer (Sigma Inc.) with protease inhibitor cocktail (Sigma Inc.) and phosphatase inhibitor cocktail (Sigma Inc.). Lysates were centrifuged at 14 000 *g* for 20 min and quantified using the bicinchoninic acid protein assay kit (Pierce Inc., Rockford, IL, USA). The same amount of protein was loaded on SDS–polyacrylamide gels and proteins were transferred onto nitrocellulose membranes. The following antibodies were used: anti-AR (Upstate Biotechnology Inc., Temecula, CA, USA), anti-ERα (Upstate Biotechnology Inc.), anti-β-tubulin (Sigma Inc.), anti-cyclin D1 (Sigma Inc.), anti-HIF-1α (BD Transduction Lab., Franklin Lakes, NJ, USA), anti-ubiquitin (Sigma Inc.) and anti-phospho-Rb and anti-PR (Cell Signaling Inc., Danvers, MA, USA). The used secondary antibodies were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA). Enhanced chemiluminescence was used to detect signal.

Immunoprecipitations

To immunoprecipitate ERα and AR, we lysed cells (10^7) with RIPA buffer. Ten microliters of anti-ERα or anti-AR (Upstate Biotechnology Inc.) were incubated with 60 μl of washed protein A agarose bead slurry (Upstate Biotechnology Inc.) in 500 μl of phosphate buffered saline (PBS) for 1 h at 4 °C. After washing the agarose beads, we diluted the cell lysate to roughly 1 μg/μl total cell protein with PBS adding 1 mg of protein from cell lysates to the reaction mixture, which was rocked gently overnight at 4 °C. Beads were precipitated and resuspended in 60 μl of 2 × Laemmli sample buffer and boiled for 5 min. Finally, we collected the beads and used the supernatant to perform SDS—polyacrylamide gel electrophoresis and immunoblot analysis as described above.

Flow cytometry

Cells were plated at a density of 30 000 cells per cm^2 per 600 μl (the same ratio as with proliferation assays). The treatments started 24–48 h after being plated. Cells were trypsinized and centrifuged at 250 *g* for 5 min, fixed in ethanol at 70%, and kept at −20 °C. Right before running the samples in the flow cytometer, the samples were stained by the addition of 1 ml of (3.4 mM $C_6H_5Na_3O_7$, Triton X-100 0.3%, propidium iodide 0.1 mg/ml, ribo-

nuclease A 20 μg/ml). The flow cytometer was a BD FACs SCAN and the analysis software was FlowJo 8.4.1.

Competitive binding assays

Peptide or Protac-AA stocks were diluted in assay buffer (50 mM *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid pH 7.5, 50 mM KCl, 20% glycerol) to 560 μM. Eleven 1:3 serial dilutions (140 μM down to 2.4 nM final assay concentration) were performed and 5 ul of each dilution was mixed with 10 ul of VHL-Elongin B-Elongin C protein complex (1 uM final concentration in assay buffer) and 5 ul of HIF-fluorescein (HIF—sequence Asp-Glu-Ala-Leu-Ala-Pro(OH)-Tyr-Ile-Pro-Asp, fluorescein added at N terminus; 12.5 nM final concentration in assay buffer). This volume was placed in a 384 well plate (Corning, Lowell, MA, USA, Catalog number 3654) in triplicate. The plate was incubated in the dark at room temperature for 1 h to stabilize the signals, and then read on an Envision plate reader (PerkinElmer, Waltham, MA, USA; polarized filters—excitation filter 480 nM, emission filters 535 nM). IC_{50} numbers determined using XLFit Excel Add-in (IDBS, Guildford, UK).

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