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A selective inhibitor of the immunoproteasome subunit LMP2 induces apoptosis in PC-3 cells and suppresses tumour growth in nude mice

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BACKGROUND: Although the proteasome is a validated anticancer target, the clinical application of its inhibitors has been limited because of inherent systemic toxicity. To broaden clinical utility of proteasome inhibitors as anticancer agents, it is critical to develop strategies to selectively target proteasomes in cancer cells. The immunoproteasome is an alternative form of the constitutive proteasome that is expressed at high levels in cancer tissues, but not in most normal cells in the body.

METHODS: To validate the immunoproteasome as a chemotherapeutic target, an immunoproteasome catalytic subunit LMP2-targeting inhibitor and siRNA were used. The sensitivity of PC-3 prostate cancer cells to these reagents was investigated using viability assays. Further, a xenograft model of prostate cancer was studied to test the *in vivo* effects of LMP2 inhibition.

RESULTS: A small molecule inhibitor of the immunoproteasome subunit LMP2, UK-101, induced apoptosis of PC-3 cells and resulted in significant inhibition (~50–60%) of tumour growth *in vivo*. Interestingly, UK-101 did not block degradation of IκBα in PC-3 cells treated with TNF-α, suggesting that its mode of action may be different from that of general proteasome inhibitors, such as bortezomib, which block IκBα degradation.

CONCLUSION: These results strongly suggest that the immunoproteasome has important roles in cancer cell growth and thus provide a rationale for targeting the immunoproteasome in the treatment of prostate cancer.

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Small molecule proteasome inhibitors have served as molecular probes as well as therapeutic agents (Joazeiro *et al*, 2006; Wehenkel *et al*, 2008). For example, lactacystin, MG-115 and epoxomicin are widely used to investigate signalling pathways regulated by the ubiquitin–proteasome system. Further, the approval of bortezomib by the FDA has validated the proteasome as a clinical target for the treatment of multiple myeloma (Adams, 2004). Bortezomib, a first-in-class proteasome inhibitor drug, is also being explored both pre-clinically and clinically as a treatment option for other malignancies, such as prostate cancer and autoimmune diseases (Genin *et al*, 2010). Although the precise molecular mechanism(s) by which bortezomib exerts its antitumour effect remains unclear, preventing activation of the NF-κB pathway is believed to be a major mechanistic basis (Hideshima *et al*, 2001; Cusack, 2003; Ling *et al*, 2003; Pham *et al*, 2003). In addition, proteasome inhibitors such as carfilzomib (Demo *et al*, 2007a), an epoxomicin-based peptide epoxyketone developed by Proteolix (now Onyx), are currently being investigated for their anticancer activity in Phase II/III clinical trials (Ikezoe *et al*, 2004; Chauhan *et al*, 2006; Joazeiro *et al*, 2006; Dreicer *et al*, 2007). Despite this promising antitumour activity, severe side effects caused by the inhibition of proteasomal and non-proteasomal activity in normal cells (Argyriou *et al*, 2008; Arastu-Kapur *et al*, 2011) are major clinical limitations of proteasome inhibitor drugs for broad applications.

Cells of haematopoietic origin express an alternative form of the proteasome known as the immunoproteasome. Additionally, inflammatory cytokines, such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), induce the synthesis of immunoproteasome catalytic subunits LMP2/β1i, MECL1/β2i and LMP7/β5i in non-haematopoietic cells, which replace their constitutive proteasome counterparts Y/β1, Z/β2 and X/β5, respectively, to form the immunoproteasome (Kloetzel, 2001). Although the function of the immunoproteasome is thought to be associated with adaptive immune responses, for example, generation of antigenic peptides and positive selection of CD8 + T cells (Murata *et al*, 2007; Nitta *et al*, 2010), increasing evidence indicates non-immune functions (Groettrup *et al*, 2010; Seifert *et al*, 2010), such as and the removal of defective ribosomal products produced by inflammatory stimuli (Seifert *et al*, 2010). Additionally, elevated levels of immunoproteasome catalytic subunits have been observed in certain disease states, such as cancer, inflammatory bowel diseases and neurodegenerative diseases (Wehenkel *et al*, 2008; Kuhn *et al*, 2011). Despite the potential for clinical application, much remains

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unknown about the functions of the immunoproteasome, prompting the development of immunoproteasome-targeting inhibitors as molecular probes as well as anticancer agents (Ho *et al*, 2007; Kuhn *et al*, 2009; Parlati *et al*, 2009). Given the low immunoproteasome expression levels in normal cells and the lack of adverse effects on immunoproteasome knockout mice (Groettrup *et al*, 2010), immunoproteasome-targeting inhibitors may have less systemic toxicity in comparison with broadly acting proteasome inhibitors such as bortezomib and carfilzomib.

In the present study, we have performed experiments using chemical and cell biological tools to validate the immunoproteasome as an antitumour target. Specifically, we report that LMP2, a catalytic subunit of the immunoproteasome, is highly expressed in several types of solid cancers, and that knockdown of LMP2 in PC-3 cells alters their growth characteristics. Moreover, we demonstrate that UK-101, an LMP2-targeting inhibitor derived from the natural product eponemycin, induces apoptosis of PC-3 cells. We also show that, unlike general proteasome inhibitors such as bortezomib, the anticancer activity of UK-101 is not mediated by blockade of NF-κB signalling in PC-3 cells. Finally, UK-101 treatment results in significant inhibition ($\sim$50–60%) of tumour growth in a mouse xenograft model of human prostate cancer with no significant phenotypic changes. Our results clearly illustrate that LMP2 inhibition provides a potential therapeutic strategy for the treatment of solid cancers.

MATERIALS AND METHODS

Cell culture

PC-3, DU 145, and LNCaP prostate cancer cell lines as well as the HeLa cervical cancer cell line were obtained from the American Type Culture Collection (Rockville, MD, USA). A549, H1155, H358, H727, H23, and H460 non-small lung cancer cell lines were a kind gift of Jong-In Park (Medical College of Wisconsin). MCF7, MDA-MB-231, and Hs 578T breast cancer cells as well as the RPMI 8226 and U266 multiple myeloma cells were obtained from the American Type Culture Collection. HCT 116, RKO, HCT-8, and HCA7 colorectal cancer cells were a kind gift of Wooin Lee (University of Kentucky). All cells were cultured according to the manufacturer's protocol in 5% CO_2 in medium containing penicillin and streptomycin (P/S) at 37 °C.

Compounds

UK-101, epoxomicin, and dihydroeponemycin were synthesised as reported (Sin *et al*, 1998, 1999; Ho *et al*, 2007). Human recombinant Tumour Necrosis Factor-α (TNF-α) was purchased from Sigma-Aldrich (St Louis, MO, USA). Interferon-γ was purchased from eBioscience (San Diego, CA, USA). Bortezomib was obtained from ChemieTek (Indianapolis, IN, USA).

Immunoblotting

Immunoblotting was performed as previously described (Ho *et al*, 2007), except for the nuclear and cytoplasmic protein extraction, which is detailed in the Supplementary Information. Antibodies were obtained from the following sources: LMP2/β1i (AbCam, Cambridge, MA, USA, Cat. No. ab78336); β-actin (Novis Biologicals, Cat. No. NB600-501); Novus (Littleton, CO, USA); GAPDH (Santa Cruz, Santa Cruz, CA, USA, Cat. No. sc-47724, Ubiquitin (Santa Cruz, Cat No. sc9133); LMP7 (AbCam, Cat No. ab3329); α-tubulin (Santa Cruz, Cat No. sc-8035); HDAC1 (Santa Cruz, Cat No. sc-8416); X (Thermo, Pittsburgh, PA, USA, Cat No. PA1-977); PARP (Biomol, Farmingdale, NY, USA, Cat No. SA-253); IκB (Cell Signaling, Danvers, MA, USA, Cat No. 9242)); phospho-IκB (Cell Signaling, Cat No. 9246); p27 (AbCam, Cat No. ab32034).

Immunohistochemistry (IHC)

Tissue sections were obtained from US Biomax (Rockville, MD, USA). An avidin-biotin immunoperoxidase assay was performed after the antigen retrieval procedure using citrate buffer, and an LMP2 antibody was used. The immune reaction was visualised using 3, 3′-diaminobenzidine (DAB), and nuclei were counter-stained with haematoxylin.

Immunofluorescence

Cells were visualised using an inverted fluorescence microscope (Nikon Ti-U microscope; Nikon Instrument INC., Melville, NY, USA) with NIS Element Research image analysis software. The confocal images were acquired using a confocal laser-scanning microscope (TCS SP2, Leica Microsystems AG, Buffalo Grove, IL, USA) at $\times$630 magnification.

siRNA

The cells were transfected with ON-TARGET*plus* siRNAs using the DharmaFECT2 siRNA transfection reagent (Thermo Fisher Scientific, Lafayette, CO, USA), according to the manufacturer's instructions. The negative control pool was human non-targeting (D-001810-10), and the test pool targeted PSMB9 (LMP2) (L-006023-00-0005).

Cell counting

Cells were mixed 1 : 1 with trypan blue, added to TC10 dual-chamber counting slides, and counted on a TC10 Automated Cell Counter (Bio-Rad, Hercules, CA, USA).

Phase-contrast microscopy

The cells were transfected with siRNA as described above. They were observed every 24 h before changing the medium and visualised using an inverted microscope (Nikon TS100 microscope) with NIS Element Research image analysis software.

Cell viability assay

PC-3 cells were plated at a density of 8000 cells per well in a 96-well plate, allowed at least 24 h to attach, and treated when 70% confluent. The percentage of viable cells was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), following the manufacturer's protocol.

Chymotrypsin-like (CT-L) cell-based activity assay

Cells were plated as described above in 96-well plates. After a 2 h treatment with inhibitors, the Proteasome-Glo Cell-Based Reagent (Promega) was added according to the manufacturer's instruction, with modified volumes (50 μl in each well instead of 100 μl). Luminescence was recorded using a Veritas Microplate Luminometer (Promega Corp., Sunnyvale, CA, USA).

Flow cytometry

Flow cytometry was performed for cell cycle analysis using assay kits (Invitrogen, Grand Island, NY, USA) following instructions provided by the manufacturers. The Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) was used according to the manufacturer's instructions.

Caspase activity assay

Caspase 3/7, 8, and 9 activities were analysed using the appropriate Caspase-Glo kit (Promega, Madison, WI, USA) for cell-based assays and following the manufacturer's instructions. Luminescence was acquired as described above.

IκB degradation assay

Cells were pretreated with inhibitor for 2 h, and then washed three times with PBS. The cells were then treated with TNF-α or vehicle in fresh medium for the indicated times. At the end of the incubation, cells were collected for immunoblotting.

Interferon-gamma treatment

PC-3 cells were treated with 100 U ml^{-1} of IFN-γ or vehicle for 24 h. At the end of the treatment, the cells were washed with PBS three times and then further cultured for indicated time periods. Cells were then collected for western blot. Alternatively, PC-3 cells were treated as described above, subcultured into 96-well plates, and treated for the MTS assay using the protocol detailed above (Cell viability assay).

Antitumour activity study in *in vivo* xenograft animal model

Six-week-old male BALB/c athymic nude mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were maintained in accordance with the National Institute of Toxicological Research of the Korea Food and Drug Administration guidelines as well as the regulations for the care and use of laboratory animals of the animal ethics committee of Chungbuk National University. PC-3 cells (1×10^7) were subcutaneously implanted into each animal. After the xenograft tumours had grown to a size of $\sim$350 mm^3, mice were dosed intraperitoneally twice a week for 3 weeks with drug or vehicle (DMSO in saline). Six mice were used in each group. When 3 weeks of treatment concluded, the animals were sacrificed by cervical dislocation. Tumours were then excised, fixed in formalin, and embedded in paraffin for IHC.

RESULTS

LMP2 is highly expressed in various types of solid cancer cell lines and primary prostate tumours

Although immunoproteasome inhibitors have been examined as potential therapeutic agents for the treatment of multiple myeloma and arthritis (Kuhn *et al*, 2009; Muchamuel *et al*, 2009; Basler *et al*, 2010; Schmidt *et al*, 2010), they have not yet been evaluated for the treatment of solid cancers. To determine the feasibility of such an approach, we first examined the expression levels of an immunoproteasome catalytic subunit, LMP2, in various cancer cell lines by western blot analysis. Many cancer cell lines expressed LMP2 at high levels, whereas normal lung fibroblasts (WI-38) had nearly undetectable LMP2 expression (Figure 1A).

To verify the clinical relevance of this LMP2 expression, matched patient samples of prostate tissues were examined. High levels of LMP2 expression were detected in the tumour samples when compared with adjacent normal tissue (Figure 1B), suggesting that the increased LMP2 expression levels may be physiologically correlated with the disease. Interestingly, our confocal fluorescence microscopic studies in PC-3 cells showed the co-localisation of LMP2 and DAPI staining, indicating the presence of LMP2 in the nucleus of PC-3 cells (Figure 1C). In contrast, previous localisation studies using HeLa cervical cancer cells (L-132) showed that, upon induction with IFN-γ, LMP2 was highly enriched in the cytosol while nuclear staining was negligible (Brooks *et al*, 2000). Subcellular fractions from PC-3 cells confirmed that a considerable amount of LMP2 is present in the nuclear enriched (NE) fraction, predominately in its catalytically active form. Although the LMP2 expression is less pronounced compared with PC-3 cells, LMP2 in LNCaP cells is found primarily in the cytosol (Figure 1C). Currently, the significance of the subcellular localisation of LMP2 in the NE fraction is not clear, but previous work suggests that the immunoproteasome may have a functional role in transcriptional regulation (Hegde and Upadhya, 2006; Kodadek, 2010). Together, these results demonstrate that LMP2 upregulation is observed in a variety of solid tumours; thus, LMP2 may provide a molecular marker and/or therapeutic target for solid cancers.

Transient LMP2 knockdown alters the growth characteristics of PC-3 cells

To verify the crucial role of LMP2 in the growth of PC-3 cells (Ho *et al*, 2007), a knockdown strategy was implemented. Specifically, PC-3 cells were transiently transfected with an siRNA pool targeted to LMP2, and the impact on PC-3 cells was examined. As shown in Figure 2A, the siRNA pool targeted to LMP2 led to undetectable levels of LMP2 expression via western blot after 72 h and continuing through 144 h post-transfection. The pooled siRNA knockdown of LMP2 also had a growth inhibitory effect, which led to a statistically significant, time-dependent decrease in the number of viable cells compared with control (Figure 2B). Compared with chemical inhibition of LMP2 by UK-101 (Ho *et al*, 2007), the effects of LMP2 knockdown on cell proliferation are modest. We suggest that, unlike UK-101, which inhibits the proteolytic activity of LMP2 without disturbing proteasome assembly or homoeostasis, LMP2 knockdown may lead to a compensatory response in which the LMP2 subunit is replaced by an alternate catalytic subunit upon proteasome assembly. Aside from differences in growth rates, no major changes in overall cell morphology were observed between the control and pooled LMP2 siRNA-treated cells (Figure 2C). This result suggests that LMP2 may have a role in the proliferation of PC-3 cells.

UK-101 treatment leads to selective and stable modification of LMP2 in PC-3 cells

UK-101 was previously shown to selectively target the LMP2 subunit of proteasomes (Ho *et al*, 2007). The covalent binding of UK-101 to LMP2 is shown by a complete upward shift of the LMP2 band in PC-3 cells upon treatment with 0.5 μM of UK-101. Conversely, covalent modification of proteasome catalytic subunits LMP7 and X by UK-101 was not observed (Figure 3A). The stability of the UK-101-LMP2 conjugate in PC-3 cells was next examined. Specifically, PC-3 cells were treated with UK-101 for 90 min, washed to remove unbound drug, and allowed to grow in the absence of drug treatment for up to 48 h. As shown in Figure 3B, the LMP2-UK-101 conjugate was stable over a 48-h period. Overall, these results demonstrate that UK-101 covalently and stably modifies LMP2 in PC-3 cells.

UK-101 inhibits the chymotrypsin-like (CT-L) activity of the proteasome in PC-3 cells

We previously reported that UK-101 inhibits the CT-L activity, but not caspase-like (C-L) or trypsin-like (T-L) activities of the purified immunoproteasome (Ho *et al*, 2007). Here, we wanted to assess these proteasomal activities in PC-3 cells treated with UK-101. As shown in Figure 3C, the CT-L activity in cell extracts from PC-3 cells treated with 1 μM of UK-101 for 2 h decreased by 55%, which is roughly in line with previous findings that LMP2 is responsible for 30–50% of the CT-L activity of the purified immunoproteasome. It should be noted that LMP7 is responsible for the residual CT-L activity ($\sim$50%) of the immunoproteasome (Ho *et al*, 2007) and that general proteasome inhibitors (epoxomicin and bortezomib) are able to remove all of the CT-L activity in these cells (Figure 3C). To confirm that this inhibition has functional consequences, the accumulation of polyubiquitinated proteins was examined over time. As shown in Figure 3D, a time-dependent accumulation of polyubiquitinated proteins was

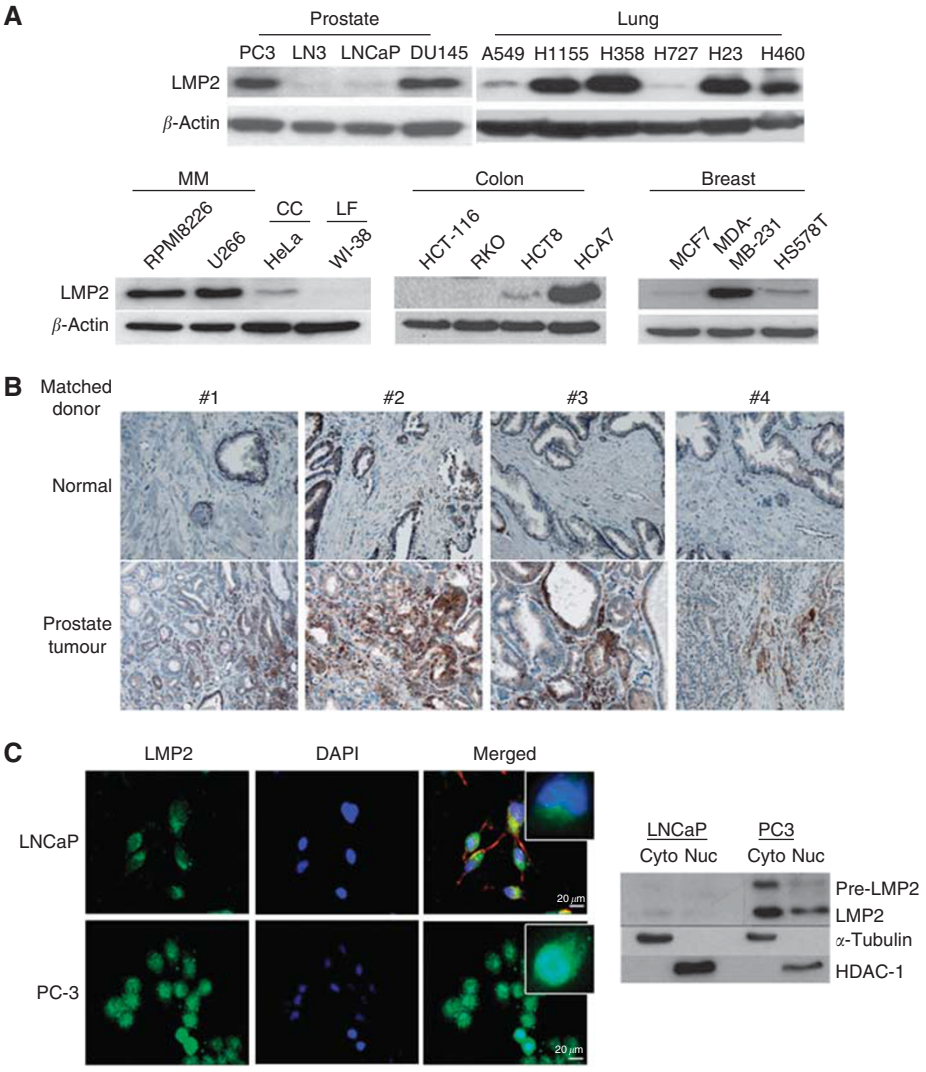


Figure 1 LMP2 is expressed at high levels in several types of cancer cell lines as well as in primary prostate tumour tissues. (**A**) Immunoblotting analysis of LMP2 expression in whole cell lysates prepared from cancer cell lines [MM = multiple myeloma; CC = cervical cancer; LF = normal human lung fibroblast). β-actin was used as a loading control. LMP2 expression is variable among cell lines, but many express detectable levels of LMP2. This subunit is generally not expressed in cells that are not of haematopoietic origin. (**B**) Immunohistochemical staining for LMP2 in normal and tumour prostate tissue sections from matched donors. Positive staining was indicated by 3, 3′-DAB (dark brown colour) and is seen at higher levels in tumour tissues. Co-staining with haematoxylin was performed, with dark blue colouring indicating the nuclei which co-localised with LMP2 in some sections. (**C**) Confocal immunofluorescence (IF) and fractionation studies using prostate cancer cell lines (ND = nuclei depleted fraction; NE = nuclei enriched fraction). In the confocal images, green staining indicates FITC conjugation with the LMP2 antibody, whereas the nuclei are stained blue with 4′, 6-diamidino-2-phenylindole (DAPI). Inset images were acquired using standard fluorescent microscopy. Unique patterns of staining are seen between LNCaP and PC-3 cells. The apparent nuclear localisation of LMP2 was confirmed by cellular fractionation. α-tubulin and HDAC1 were used as fractionation controls.

observed in cells treated with UK-101. These results suggest that UK-101 induces accumulation of polyubiquitinated proteins, resulting in disturbance of cellular protein homoeostasis.

UK-101 induces cell cycle arrest and subsequent apoptosis of PC-3 cells

We previously demonstrated that a 48-h treatment of PC-3 cells with UK-101 leads to inhibition of cell growth (Ho *et al*, 2007). Thus, an examination of whether this effect is due to apoptotic cell death was undertaken. Epoxomicin, a potent and broadly acting proteasome inhibitor, was used as a positive control in these apoptotic assays. Following a 24-h incubation with UK-101, the cells appeared to undergo cell cycle arrest in the G1/S phase in a dose-dependent manner (Supplementary Figure 1A). However, no cell death was observed. To confirm this, annexin V/PI staining and flow cytometry analysis was used. Again, no apparent increase in apoptotic cells was observed after a 24-h treatment with UK-101 (Supplementary Figure 1B). Accumulation of p27 (Supplementary Figure 1C), a CDK inhibitor associated with progression of cell cycle during the G1/S phase, further supported UK-101-induced G1/S cell cycle arrest. Together, these results demonstrate that treatment with UK-101 for 24 h causes cell cycle arrest but not apoptosis. However, it is unclear how UK-101 induces the G1/S phase arrest.

Next, similar experiments were undertaken at the 48 h time point utilised in previous cell viability assays. Our data indicate that cells arrest in the G2/M phase (Figure 4A) and undergo

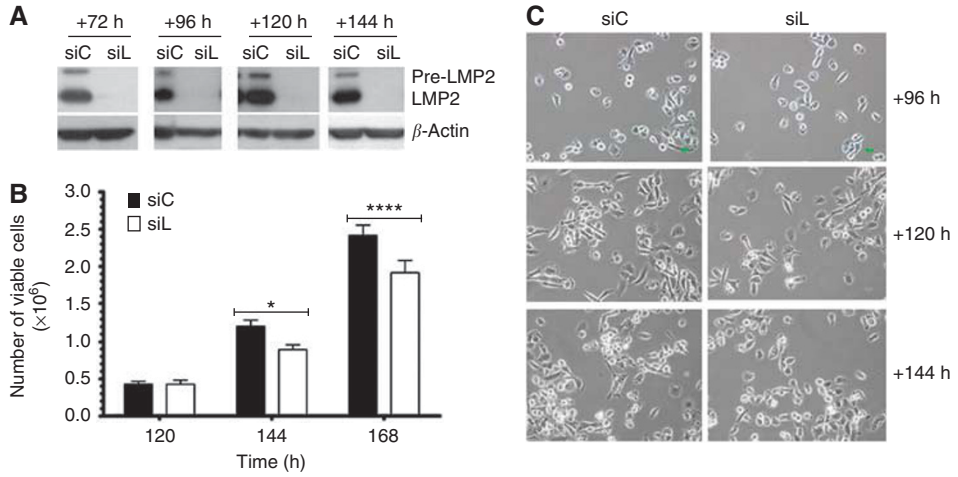


Figure 2 LMP2 knockdown in PC-3 cells decreases cell viability over time. (**A**) Time-dependent knockdown of LMP2. After 72 h, a significant reduction in LMP2 expression was seen in cells transiently transfected with siRNA targeting LMP2 when compared with cells transfected with scrambled control siRNA. (**B**) The siRNA targeted to LMP2 slows cell proliferation. Viable cell counts were obtained using a TC10 automated cell counter (Bio-Rad). The experiments were repeated at least four times, and the numbers of viable cells were averaged and plotted. Significance was determined using a two-way ANOVA (*$P<0.05$, ****$P<0.001$). (**C**) Phase-contrast microscopy of cells treated with siRNA targeted to LMP2. Although differences in total cell numbers were observed, there was no obvious phenotypic change seen in cells treated with siRNA against LMP2 when compared with cells treated with scrambled control siRNA.

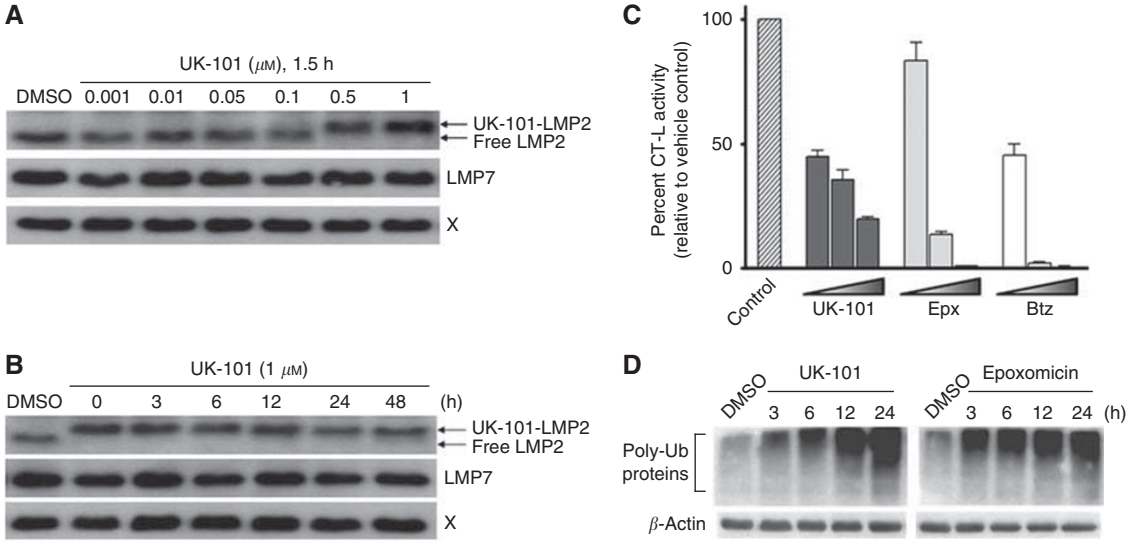


Figure 3 UK-101 selectively inhibits LMP2 and its catalytic activity via a stable covalent modification. (**A**) Following a 90 min incubation of cells with UK-101, the concentration dependence of UK-101 binding to LMP2 in PC-3 cells was examined via western blotting. The increase in apparent molecular weight of LMP2 indicates its covalent modification by UK-101. No mobility changes in the X subunit of the constitutive proteasome or the LMP7 subunit of the immunoproteasome were observed. (**B**) The stability of the covalent modification of LMP2 with UK-101 was examined following removal of excess-free UK-101 from the system. PC-3 cells were treated with drug or vehicle for 90 min, then washed and given fresh media. Samples were collected at the indicated times and subjected to western blotting. The binding of UK-101 to LMP2 is maintained for at least 48 h. (**C**) Inhibition of the chymotrypsin-like (CT-L) activity of cellular proteasomes. A proteasome activity assay was performed with PC-3 cells treated with increasing concentrations of proteasome inhibitors (UK-101: 1, 10, 50 μM; epoxomicin or bortezomib: 5, 50, 500 nM) for 2 h. The CT-L activity of total cellular proteasomes was measured using the CT-L luminogenic substrate Suc-Leu-Leu-Val-Tyr-aminoluciferin. Values were obtained in triplicate, and error bars represent standard deviations. (**D**) Cells were treated with inhibitors, lysed, and probed for ubiquitin. Both UK-101 (25 μM) and epoxomicin (1 μM) caused an accumulation of polyubiquitinated proteins that increased over time.

apoptosis (Figure 4B) after a 48-h incubation with UK-101. This was further verified by the observed cleavage of poly (ADP-ribose) polymerase (PARP) and activation of caspases 3/7, 8 and 9 (Figure 4C and D). Taken together, our results indicate that UK-101 induces apoptosis via activation of both the intrinsic and extrinsic pathways.

The antitumour activity of UK-101 is independent of inhibiting NF-κB activation in PC-3 cells

Several studies have shown that the transcription factor nuclear factor-kappa B (NF-κB) has a pivotal role in the protection of cancer cells from proteasome inhibitor-induced apoptosis

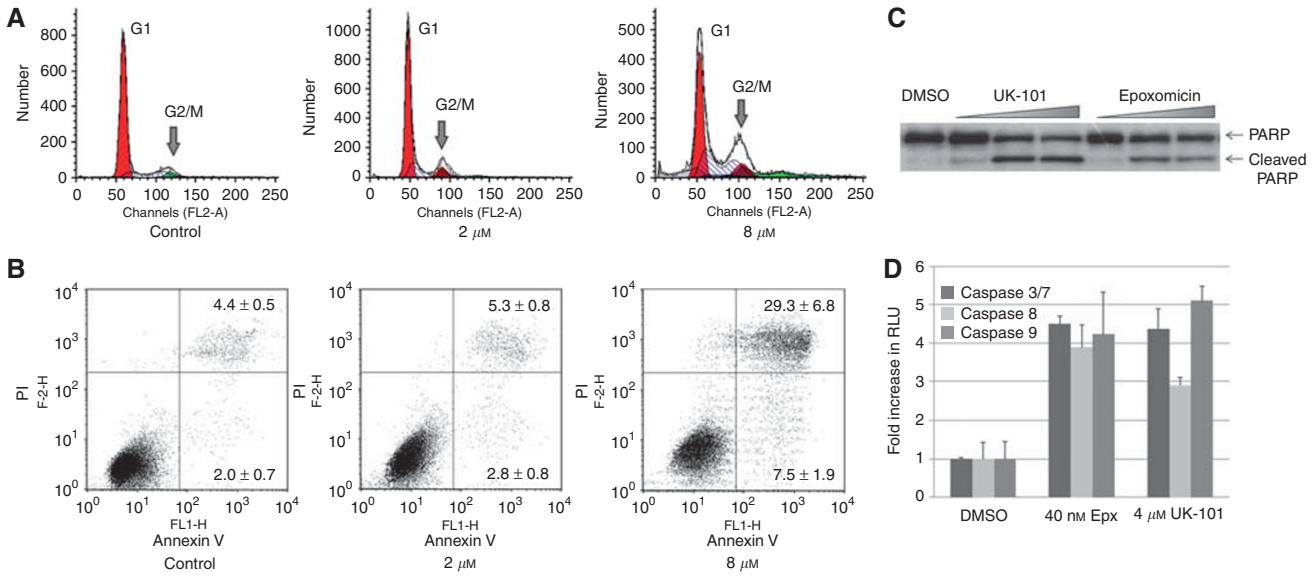


Figure 4 UK-101 induces cell cycle arrest and apoptosis in PC-3 cells. (**A**) After a 48-h treatment with UK-101, the cell cycle distribution was analysed using flow cytometry. A dose-dependent increase in cells in the G2/M phase of the cell cycle was observed. [%CV was between 6.80 and 8.65 for all conditions]. (**B**) Apoptosis analysis was conducted after a 48-h treatment with UK-101 using Propidium Iodide (PI) and Annexin-V (AV) staining. The percentages of cells in early (AV positive & PI negative) and late (AV & PI positive) stages of apoptosis were determined by flow cytometry. Numbers are means plus or minus standard deviations. (**C**) Poly (ADP-ribose) Polymerase (PARP) cleavage was examined, as caspase-3 cleaves PARP during the apoptotic cascade, following a 48-h treatment with UK-101 or epoxomicin. PARP cleavage was seen in a dose-dependent manner with both inhibitors (UK-101: 1, 2, 8 μM; epoxomicin or bortezomib: 5, 50, 500 nM), indicating apoptotic cell death. (**D**) Caspase (3/7, 8, and 9) activity assays were performed in PC-3 cells following a 48-h treatment with UK-101 or epoxomicin to confirm caspase-mediated apoptosis. Luminescence was normalised with that of control, and error bars represent standard deviations. Significant induction of caspase activity was observed for epoxomicin- and UK-101-treated cells ($P \leqslant 0.001$ when compared with DMSO using one-way ANOVA), suggesting apoptotic cell death.

(Rayet and Gelinas, 1999; Baldwin, 2001). General proteasome inhibitors such as bortezomib block the degradation of the NF-κB inhibitory protein IκBα. The sensitivity of PC-3 cells to bortezomib-induced apoptosis is reportedly due in part to the inhibition of NF-κB activation (Fahy *et al*, 2005; Ayala *et al*, 2008). With this in mind, we examined whether inhibition of NF-κB is involved in UK-101-induced apoptosis in PC-3 cells. It should be noted that the role of LMP2 in IκBα degradation/NF-κB nuclear translocation has been controversial over the years (Hayashi *et al*, 2000; Kessler *et al*, 2000; Runnels *et al*, 2000). TNF-α was used to stimulate phosphorylation and degradation of IκBα. Interestingly, although a 0.5 μM dose of UK-101 completely modifies LMP2 (Figure 3A and B), even a 50-μM dose of UK-101 did not inhibit the degradation of phosphorylated IκBα (Figure 5). This suggests that LMP2 may not be involved in IκBα degradation. We therefore hypothesise that UK-101 leads to the apoptosis of PC-3 cells via molecular mechanisms independent of NF-κB.

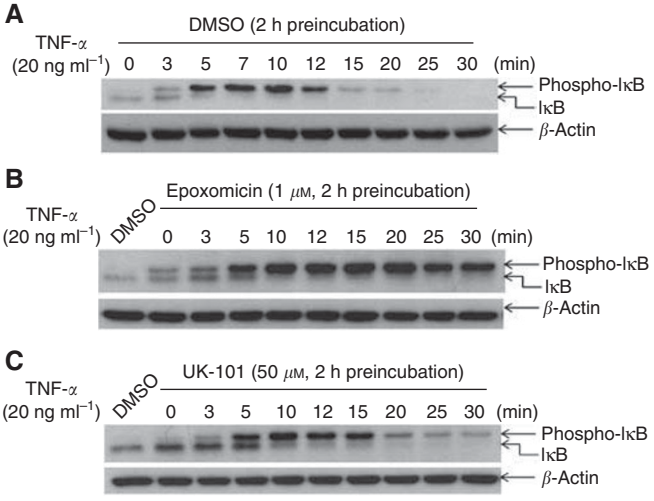
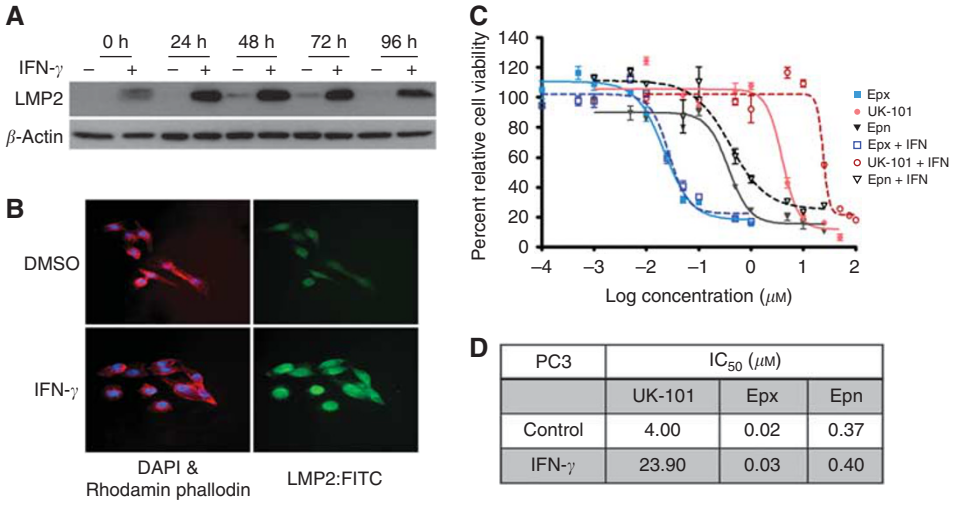


Figure 5 UK-101 does not block the degradation of phosphorylated IκBα in cells treated with TNF-α. After a 2-h pre-incubation of PC-3 cells with DMSO, epoxomicin, or UK-101, the cells were repeatedly washed to remove excess compound. TNF-α was then added, and the phosphorylation and degradation of IκBα was examined by western blot. (**A**) TNF-α-induced phospho-IκBα is rapidly degraded. (**B**) The broadly acting proteasome inhibitor epoxomicin blocks degradation of phospho-IκBα. (**C**) UK-101 does not inhibit degradation of TNF-α-induced phospho-IκBα.

LMP2 upregulation decreases the sensitivity of PC-3 cells to UK-101-induced apoptosis

We next investigated whether LMP2 upregulation alters the sensitivity of cancer cells to UK-101-induced inhibition of cell growth. PC-3 cells were treated with IFN-γ, which has been shown to induce LMP2 expression (Heink *et al*, 2005; Strehl *et al*, 2005). We hypothesised that cells treated with an LMP2-inducer would be less sensitive to the drug, as a greater concentration of drug would be required to achieve the same percentage of protein inhibition. As shown in Figure 6A, cells pretreated with 100 U ml^{-1} of IFN-γ for 24 h expressed significantly higher levels of LMP2 for up to 96 h after IFN-γ was removed, as compared with vehicle (DMSO) treatment. It should be noted that PC-3 cells express high levels of LMP2 compared with normal cells and many tumour cells (Figure 1A). In addition, IFN-γ treatment did not change the cellular distribution pattern of LMP2, as shown via immunofluorescence (Figure 6B).



PC3	IC_{50} (μM)		
	UK-101	Epx	Epn
Control	4.00	0.02	0.37
IFN-γ	23.90	0.03	0.40

Figure 6 INF-γ-induced overexpression of LMP2 decreases UK-101 efficacy. (**A**) Interferon gamma (IFN-γ) treatment upregulates the expression of LMP2 in PC-3 cells. Cells were pre-treated with 100 U ml^{-1} of IFN-γ or vehicle (DMSO) for 24 h, washed, and allowed to grow for the indicated times. Lysates were subjected to western blotting and showed a large and sustained upregulation of LMP2 by IFN-γ. (**B**) The localisation of IFN-γ-induced LMP2 follows the same general pattern as constitutive LMP2 expression in PC-3 cells. After a 24 h pretreatment with IFN-γ (250 U ml^{-1}) or vehicle (DMSO), cells were examined via immunofluorescence microscopy. The LMP2 antibody was conjugated to Alexa Fluor 488 (green), rhodamin phallodin was used as an f-actin stain (red), and 4′, 6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei (blue). (**C**) Upregulation of LMP2 significantly changed the sensitivity of cells to UK-101, but not to epoxomicin (Epx) and eponemycin (Epn). PC-3 cells pretreated with IFN-γ are indicated by dashed lines and open symbols, while those pretreated with vehicle control (DMSO) have solid lines and symbols. Data were graphed in GraphPad Prism™ using non-linear regression (sigmodial dose response (variable slope)) and error bars represent the standard error of the mean. (**D**) IC_{50} values derived from the graphs in **C**. The change in eponemycin falls within the 95% confidence interval.

As these treatment conditions allowed elevated expression levels of LMP2 to be maintained over 72 h, we combined this paradigm with the MTS assay to examine the effect of increased LMP2 levels on cell viability. Cells were pretreated with IFN-γ for 24 h, subcultured into 96-well plates, and allowed to attach for 24 h. The cells were then treated with proteasome inhibitors for 48 h, and cell viability was analysed via the MTS assay. Following pretreatment with IFN-γ, the cells became markedly less sensitive to UK-101 (Figure 6C and D), while their sensitivity to general proteasome inhibitors (epoxomicin and dihydroeponemycin) remained unchanged. Given this unaltered sensitivity to general proteasome inhibitors, the significant decrease in UK-101 sensitivity is most likely attributed to increased expression of LMP2, the selective target of UK-101, rather than to other intracellular changes caused by IFN-γ treatment. These results support the hypothesis that the pharmacological effect of UK-101 is mediated through LMP2 and are in line with the results from LMP2 knockdown studies shown in Figure 2. Additionally, these results strongly indicate that the immunoproteasome has a crucial role in PC-3 cell growth.

UK-101 inhibits tumour growth in a mouse xenograft model of prostate cancer

The results described above clearly illustrate that UK-101 induces apoptosis of PC-3 cells and suggest this is primarily due to the inhibition of LMP2. Next, tumour xenograft model studies were conducted to examine the *in vivo* effectiveness of UK-101. To determine whether UK-101 inhibits tumour growth *in vivo*, we subcutaneously implanted PC-3 cells in 6-week-old male BALB/c athymic nude mice. When the tumours became palpable (~350 mm^3), the mice were treated intraperitoneally with either vehicle control (DMSO in saline), cisplatin (2 mg kg^{-1}), or UK-101 (1 or 3 mg kg^{-1}) twice a week for 3 weeks. These cisplatin and UK-101 concentrations were chosen on the basis of other reports demonstrating the antitumour effects of cisplatin (Lee *et al*, 2009) and carfilzomib (Demo *et al*, 2007b). Tumour volume was measured twice a week over the course of the 3-week drug treatment. Similar to the *in vitro* data, significant inhibition (~50%) of tumour growth by UK-101 in comparison with vehicle was observed after 14 days (Figure 7A). This UK-101-induced inhibition of tumour growth was further confirmed by the weights and sizes of the excised tumours, which were similar to those of cisplatin-treated animals (Figure 7B). Importantly, in contrast to mice treated with cisplatin, the UK-101-treated mice appeared to suffer significantly less systemic toxicity (Supplementary Figure 2A) and the weights of mice treated with UK-101 remained steady over the 3-week treatment period (Supplementary Figure 2B). To determine whether the inhibition of *in vivo* tumour growth by UK-101 is due to apoptosis of the tumour cells, mice were euthanised and tumours were isolated for IHC analyses. IHC revealed increased staining of cleaved caspase 3 in the UK-101-treated tumour tissue samples when compared with vehicle control (Figure 7C). Together, these results indicate that UK-101 inhibits tumour growth in a mouse xenograft model of human prostate cancer by inducing apoptosis.

DISCUSSION

The field of immunoproteasome biology will benefit from the development and validation of small molecule inhibitors of catalytic functions. Both LMP2 (Ho *et al*, 2007) and LMP7 (Muchamuel *et al*, 2009; Parlati *et al*, 2009) targeting inhibitors have now been reported, allowing for complementary investigations of immunoproteasome function *in vitro* and *in vivo*. In this study, using both an LMP2-targeting small molecule inhibitor

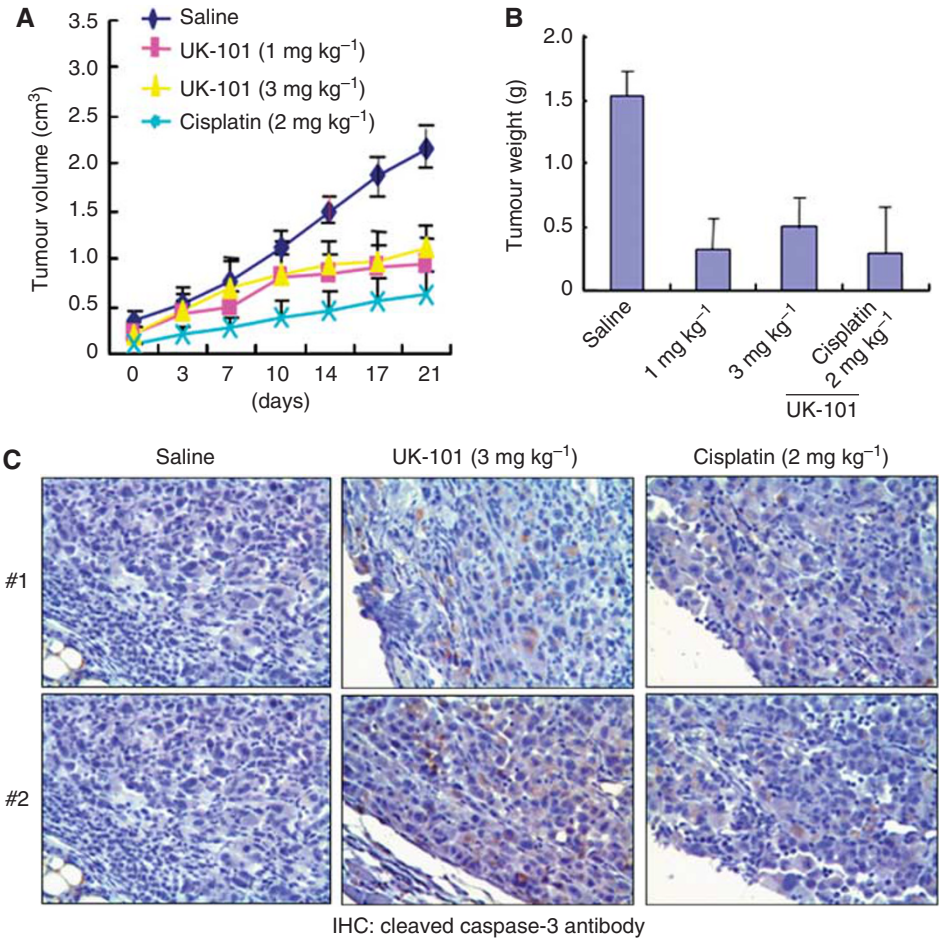


Figure 7 UK-101 shows antitumour effects *in vivo*. Male nude mice bearing PC-3 tumours were treated with either saline (0.05% DMSO), UK-101 (1.0 or 3.0 mg kg^{-1}), or cisplatin (2 mg kg^{-1}) twice a week for 3 weeks. Each experimental group contained six mice and error bars represent the standard deviation of the mean. (**A**) Tumour growth inhibition by UK-101. Tumour volumes were measured with vernier calipers and calculated using the following formula: $(A \times B^2)/2$, where A is the larger and B is the smaller of the two dimensions **B**. At the end of the treatment time (21 days), mice were euthanised and tumours were isolated. The masses of these tumours were measured and averaged. (**C**) Three samples from each group were isolated and subjected to immunohistochemical analysis for cleaved caspase-3. Positive staining (DAB) is indicated by a brown colour, whereas counterstaining with haematoxylin shows nuclei as blue.

(UK-101) and siRNA, we show that the LMP2 subunit is a potential target for the treatment of solid cancers *in vivo*.

We found that a variety of solid cancer cell lines as well as patient prostate tumour tissues express high levels of LMP2. Given that LMP2 upregulation is implicated in several pathological disorders and that UK-101 inhibits the growth of prostate cancer cells (Fitzpatrick *et al*, 2007; Wehenkel *et al*, 2008), LMP2 may represent a novel drug target. Our knockdown studies indicate that depletion of the LMP2 subunit in PC-3 cells has a growth inhibitory effect, further supporting LMP2 as a novel target. This may be attributed to an altered immunoproteasome assembly using its constitutive counterparts in the LMP2 knockdown cells, leading to the formation of intermediate immunoproteasomes lacking LMP2 (Guillaume *et al*, 2010). Likewise, knockdown or knockout approaches to prevent expression of proteasome subunits may suffer from the confounding effects of losing both the protein's functionality as an enzyme and its role as a scaffolding protein during proteasome assembly, thereby disrupting immunoproteasome homoeostasis. Use of the LMP2-targeting inhibitor UK-101 will prove useful in demonstrating that chemical inhibition of LMP2 activity may provide a therapeutic benefit in solid cancers.

In this study and in our previous studies (Ho *et al*, 2007), the selectivity of UK-101 towards LMP2 was demonstrated by mobility shift assays. Our apoptosis assay data showed that UK-101 induces apoptosis of PC-3 cells via activation of both the intrinsic and extrinsic pathways (Figure 4). This result is consistent with previous observations made of bortezomib or epoxomicin-induced apoptosis (Seki *et al*, 2010). We also demonstrated that UK-101 induces accumulation of poly-ubiquitinated proteins *in vitro* and detection of cleaved caspase-3 *in vivo*, resulting in apoptosis and suppression of tumour growth, respectively.

Additionally, to investigate whether cancer cell growth inhibition correlates with the inhibition of proteasomal proteolytic activity, we measured the catalytic activity of PC-3 cells treated with UK-101. Indeed, this treatment inhibited a significant portion (>50%) of the CT-L activity in these cells in a concentration-dependent manner, demonstrating UK-101's ability to effectively inhibit the catalytic activity of the proteasome in cells. Of note, we previously reported that LMP7 and LMP2 are responsible for ~50–70% and ~30–50% of the CT-L activity of the purified immunoproteasome, respectively (Ho *et al*, 2007).

Considering this, it is intriguing that selective inhibition of LMP7 by small molecules, such as PR-957, did not cause

accumulation of poly-ubiquitinated proteins and subsequently showed no significant effect on cell viability (Muchamuel *et al*, 2009; Parlati *et al*, 2009). Similarly, it has been reported that selective inhibition of X/β5 or LMP7 alone is not sufficient to induce an antitumour effect in multiple myeloma cells (Parlati *et al*, 2009). Combined with these studies, our data obtained from LMP2 knockdown studies suggest that LMP2 may have a distinct functional role in cells, and that this may provide unique therapeutic indications for inhibitors of each of these subunits. Previously, similar observations about distinct roles of individual catalytic subunits of the constitutive proteasome have been made (Kisselev *et al*, 2006).

Another interesting observation is that UK-101 did not block TNF-α-induced IκBα degradation (Figure 5), in contrast to broadly acting proteasome inhibitors such as bortezomib and carfilzomib. As the activation of NF-κB pathways is an important component of cell survival processes, it is believed that one of the major mechanisms by which proteasome inhibitors induce apoptosis is the inhibition of the NF-κB activation (Cortes Sempere *et al*, 2008; Shen and Tergaonkar, 2009; Yu *et al*, 2009). More specifically, in PC-3 cells, NF-κB is hyper-activated, has a major role in cell survival, and its inhibition is considered to be a major mechanism by which general proteasome inhibitors exert their antitumour activity (An *et al*, 2003). Thus, it is intriguing that UK-101 does not inhibit degradation of IκBα, leading to the hypothesis that UK-101 may cause apoptosis in PC-3 cells via an NF-κB-independent pathway. In this regard, it is worth mentioning that an aldehyde pharmacophore-based LMP2 inhibitor was shown to induce accumulation of both IκBα and ubiquitinated IκBα in multiple myeloma RPMI 8226 cells (Kuhn *et al*, 2009). Considering the inherent activity of the aldehyde pharmacophore, it is unclear how selective this aldehyde inhibitor is for LMP2 over other non-proteasomal proteases.

Finally, it is noteworthy that UK-101-treated animals appear to suffer no significant toxic effects, as evidenced by their maintained weights over the treatment period, in contrast to cisplatin-treated animals (Supplementary Figure 2). In line with our observation, aside from defects in responding to certain types of viral infections, LMP2 knockout produced no significant side effects in animal models (Arnold *et al*, 1992; Yewdell *et al*, 1994; Nussbaum *et al*, 2005; Yewdell, 2005). Taken all together, we envision that an LMP2-targeting approach may provide a novel antitumour strategy with less systemic toxicity than conventional antitumour agents.

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