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Physalin B, a novel inhibitor of the ubiquitin-proteasome pathway, triggers NOXA-associated apoptosis

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ABSTRACT

The ubiquitin-proteasome pathway plays a critical role in the degradation of proteins involved in tumor growth and has therefore become a target for cancer therapy. In order to discover novel inhibitors of this pathway, a cellular assay reporter of proteasome activity was established. Human DLD-1 colon cancer cells were engineered to express a 4 ubiquitin-luciferase (DLD-1 4Ub-Luc) reporter protein, rapidly degraded via the ubiquitin-proteasome pathway and designed DLD-1 4Ub-Luc cells. Following treatment with reference proteasome inhibitors, the 4Ub-Luc protein accumulated in DLD-1 4Ub-Luc cells and a 80-fold increase in luciferase-produced bioluminescence signal was measured, as compared to untreated cells. The screening of over 30,000 compounds using this DLD-1 4Ub-Luc assay led to the identification of physalin B as a novel inhibitor of the ubiquitin-proteasome pathway. Indeed, physalin B induced an increase in bioluminescence from DLD-1 4Ub-Luc cells, at concentrations also producing an accumulation of ubiquitinated proteins and inhibiting TNFα-induced NF-κB activation. Physalin B did not inhibit catalytic activities of purified proteasome and interfered with cellular proteasomal catalytic activities at 4- to 8-fold higher concentrations than that required to induce significant increase in bioluminescence and accumulation of ubiquitinated proteins in DLD-1 4Ub-Luc cells. Furthermore, physalin B proved to be cytotoxic, triggered apoptosis in DLD-1 4Ub-Luc cells and induced the pro-apoptotic protein NOXA, characteristic of the proteasome signaling pathway. Therefore, the use of the DLD-1 4Ub-Luc assay allowed the identification of a novel inhibitor of the ubiquitin-proteasome pathway that might interfere with proteasome functions in a different way from reference proteasome inhibitors.

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Abbreviations: ATCC, American type cell collection; DEVD-MR, Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-Magic Red; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FBS, fetal bovine serum; MEM, minimal essential medium; RLU, relative light unit; TNFα, tumor necrosis factor; Ub, ubiquitin.

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1. Introduction

In addition to removing damaged and unneeded proteins, proteasome-mediated proteolysis is a mechanism for controlling important regulatory proteins within cell [1,2]. Proteins destinated for proteolysis are tagged by the attachment of a polyubiquitin chain and subsequently degraded by the 26S proteasome. The 26S proteasome is a large multi-unit complex composed of a central 20S catalytic core and two 19S regulatory caps, present in the cytoplasm and the nucleus of all eukaryotic cells. The 20S core particle is a cylindrical structure containing the three main catalytic activities of the proteasome, namely chymotrypsin-like, trypsin-like and caspase-like activities [3]. The proteasome has recently emerged as an important target for anticancer therapy [4], as illustrated by the clinical efficacy of the dipeptidylboronic acid bortezomib (Velcade), a potent and specific inhibitor of the proteasome, approved for the treatment of multiple myeloma [5,6]. Almost all the synthetic and natural inhibitors of the proteasome act predominantly on the chymotrypsin-like activity and have, usually much weaker, effects on the two other sites [7]. Indeed, screening for proteasome inhibitors has often been based on chymotrypsin-like activity measurement using purified proteasome and fluorogenic synthetic peptide substrates. This experimental set-up does not reproduce the complex interactions leading to ATP-dependent degradation of ubiquitinated proteins and does not assess the influence of vital parameters, such as bioavailability and cell permeability, that may affect the therapeutic value of proteasome inhibitors. Previous studies demonstrated that fusion of ubiquitin to firefly luciferase, β-lactamase or green fluorescent protein constitutes a dynamic reporter of function and inhibition of the 26S proteasome in cultured cells [8–10]. Indeed these reporters were degraded rapidly under steady-state conditions and stabilized in a concentration- and time-dependent manner in response to proteasome inhibitors. These systems allow rapid quantification of ubiquitin–proteasome activity in living cells. We have exploited these properties and design an ubiquitin-luciferase fusion protein-based screening assay. More specifically, the stably transfected human DLD-1 colon cancer cells expressing the 4 ubiquitin-luciferase fusion protein (DLD-1 4Ub-Luc) were used for screening and evaluation of proteasome inhibitors. This cellular assay proved sufficiently robust, specific and reproducible to be used for high throughput screening to identify modulators of proteasome activity [11]. A total of 15,744 extracts or fractions from plant collections and 18,816 molecules from chemical libraries were screened for their capacity to stabilize the 4Ub-Luc reporter in DLD-1 4Ub-Luc cells [11]. This led to 66 hits amongst which physalin B was identified from a methanol extract of aerial parts of the plant *Physalis angulata*.

The purpose of the present study was to characterize the proteasome inhibitory properties of physalin B and to further investigate its pharmacological activities. The adequacy of the DLD-1 4Ub-Luc assay to screen for novel inhibitors of the ubiquitin-proteasome pathway was first described and the capacity of physalin B to stabilize the 4Ub-Luc reporter protein in DLD-1 4Ub-Luc cells was confirmed using the non-automated assay. Then, in order to further support evidence for proteasome inhibition by physalin B, its effects on the level of ubiquitinated proteins in DLD-1 4Ub-Luc cells, on the catalytic activities of purified or cellular proteasome, and on TNFα-induced NF-κB activation were examined. The capacity of physalin B to induce the proapoptotic protein NOXA, to trigger apoptosis and to display cytotoxicity in human cancer cells was also investigated.

2. Materials and methods

2.1. Compounds

The following compounds were obtained from various sources as indicated: epoxomicin from Boston Biochem (USA), MG-262 from Calbiochem (France), lactacystin, clasto-lactacystin β lactone from Sigma–Aldrich (Lyon, France), bortezomib, monastrol and etoposide, synthesized in Pierre Fabre Laboratories (Castres, France), were all solubilized in DMSO to achieve a concentration of 0.1% in the final reaction volume.

Physalin B was extracted in methanol from the aerial part of the plant *P. angulata*, as previously described [11]. *P. angulata* is a common annual herb found in many parts of the tropics (Africa, Asia and the Americas). Physalin B structure was identified by mass spectrometry and nuclear magnetic resonance experiments and by comparison with analytical data from the literature [11–13]. For the present studies, physalin B was solubilized in DMSO to achieve a concentration of 0.1% in the final reaction volume.

2.2. Generation of stably transfected DLD-1 4Ub-Luc and DLD-1 Luc cell lines

The coding region of ubiquitin (Ub) was amplified by RT-PCR with HeLa cDNA to introduce 5′ HindIII-NcoI-SpeI and 3′ SacII-BspHI-XbaI restriction sites. The XbaI site allows the mutation of glycine 76 to valine in ubiquitin, in order to limit its cleavage by ubiquitin hydrolase. The PCR product was digested with HindIII and SacII and ligated into pBluescript II vector (Stratagene, Amsterdam, The Netherlands). This plasmid was then digested with HindIII and BspHI, and the Ub^{G76V} containing fragment was isolated and fused to the N-terminus of firefly luciferase in pGL-3 vector opened with HindIII and NcoI. Various multimerized forms of Ub^{G76V} (one, two or four Ub^{G76V}) fused to firefly luciferase were generated using NcoI/BspHI and SpeI/XbaI compatibility. The four Ub^{G76V}-firefly luciferase fusion fragment, selected to establish the stably transfected cell line, was then excised from pGL-3-4Ub^{G76V} plasmid using HindIII and XbaI and cloned in the bicistronic pRCEN1 vector, previously described [14], between the CMV and the IRES-neomycin cassette. The wild type firefly luciferase excised from pGL-3 was also cloned in the pRCEN1 vector. The resultant four Ub^{G76V}-firefly luciferase fusion and wild type firefly luciferase constructs were designated 4Ub-Luc and Luc, respectively. These two vectors were used to stably transfect the human DLD-1 colon cancer cells, using the Lipofectamine technique (Invitrogen, USA) followed by selection in 0.8 mg/ml G418 (Invitrogen, USA), to generate the DLD-1 4Ub-Luc and DLD-1 Luc cell lines.

2.3. Cell lines and culture

The human DLD-1 colon cancer cells were purchased from the American Type Cell Collection (Rockville, USA). The engineered DLD-1 4Ub-Luc and DLD-1 Luc cells were cultured in MEM medium (Gibco, France), supplemented with 5% heat-inactivated FBS (Gibco), 2 mM glutamine, 1.25 μg/ml fungizone (Gibco) and 50 μg/ml penicillin/streptomycin (CambreX, France).

2.4. Measurement of bioluminescence from DLD-1 4Ub-Luc and DLD-1 Luc cells

DLD-1 4Ub-Luc or DLD-1 Luc cells were seeded at 10,000 cells/well in white 96-well plates and incubated with test compounds or drug solvent for 2, 4, 6, 8, 16 or 24 h, at the concentrations indicated for individual experiments. Luciferase activity in cell lysates was determined with a luciferase assay kit (Promega, France) and luminescence was red using a LB 960 Centro luminometer (Berthold, France).

2.5. Western blotting analyses

Following treatment with the indicated test compounds for the indicated times, lysates of DLD-1 4Ub-Luc cells were fractionated by 4–20% acrylamide SDS-PAGE and transferred onto polyvinylidene difluoride membrane for assessment of the levels of ubiquitinated proteins, p27 or NOXA. After blocking non-specific sites with a solution containing 5% free-fat milk and 5% FCS, the transfer membrane was probed overnight with an anti-ubiquitin (Santa Cruz, Germany), an anti-p27 (Beckton Dickinson, France), an anti-PARP (Beckton Dickinson, France) or an anti-NOXA (Calbiochem, France) antibody, followed by a 2-h incubation with a goat anti-mouse secondary antibody conjugated to peroxidase (Jackson Immuno-Research, USA). Proteins of interest were visualized by enhanced chemoluminescence (Pierce, Rockford, Il, USA).

2.6. Purification of human proteasome

Proteasome was partially purified according to previous reports [15]. All steps were conducted at +4 °C, and solutions were buffered at pH 7.5. A pellet of 5×10^9 human HeLa cells (4C, Belgium), was lysed in 2 pellets volume buffer containing: 25 mM Hepes, 1 mM DTT, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.5% NP40, 1 mM ATP, 100 μM Na_3VO_4. This lysate was frozen 10 min at −80 °C before centrifugation for 3 h at 100,000 g. The supernatant was diluted twice in buffer A: 10% glycerol, 30 mM Tris–HCl, 1 mM ATP, 5 mM $MgCl_2$, 5 mM NaF, 2 mM DTT, 1 mM EDTA, 100 μM Na_3VO_4, to which 10 mM NaCl was added. This sample was loaded, at a flow rate of 0.7 ml/min, onto a 70 ml DEAE column (Amersham Biosciences). The column was washed by 5 column volumes buffer A + 10 mM NaCl, then 5 column volumes buffer A + 100 mM NaCl. Proteins were then eluted with 5 column volumes of a 100 mM to 300 mM NaCl gradient in buffer A at a flow rate of 2 ml/min. Fractions of 5 ml were collected for subsequent protein quantitation and *in vitro* evaluation of proteasome activity. Fractions containing at least 50% of the maximal activity observed were pooled and separated on a Heparine Sepharose column. The pool from the DEAE column was first dialysed against buffer H: 10% glycerol, 50 mM Hepes, 1 mM ATP, 5 mM MgCl2, 5 mM NaF, 1 mM DTT, and 100 μM Na_3VO_4, then loaded, at a flow rate of 0.2 ml/min, onto a 10 ml Heparin Sepharose column (Amersham Biosciences). The column was then washed, at a flow rate of 0.5 ml/min, with 5 column volumes of buffer H, and proteins were eluted with 5 column volumes of a 0 M to 1.2 M NaCl gradient in buffer H. Fractions of 5 ml were collected for subsequent protein quantitation and *in vitro* evaluation of proteasome activity. Fractions containing at least 50% of the maximal activity observed were pooled and glycerol was added to reach 20% final before freezing at −80 °C.

2.7. Inhibition of purified proteasome activity

The fluorogenic substrates methoxysuccinyl-Succ-Leu-Leu-Arg-aminomethylcoumarin, Z-Leu-Leu-Glu-aminomethylcoumarin, or Succinyl-Leu-Leu-Val-Tyr- aminomethylcoumarin (NeoSystem, Strasbourg, France) were used to measure trypsin-like, caspase-like or chymotrypsin-like proteasome catalytic activities, respectively, as previously reported [16]. Assays were carried out in a 200 μl reaction buffer (30 mM Tris–HCl pH7.5, 1 mM ATP, 5 mM MgCl2, 5 mM NaF, 1 mM DTT, and 100 μM Na_3VO_4) containing 100 μM of one of the fluorogenic substrates and 3–9 μg human purified proteasome (the amount of purified proteasome was adjusted to get similar catalytic activity with the three substrates), in the presence of indicated proteasome inhibitors at different concentrations or in drug solvent for 90 min at 37 °C. The cleavage of fluorogenic peptide was determined by monitoring the fluorescence of released aminomethylcoumarin using a spectrofluorimeter (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 395 nm and an emission wavelength of 460 nm.

2.8. Inhibition of cellular proteasome activity in DLD-1 4Ub-Luc cells

DLD-1 4Ub-Luc cells were seeded at 10^4 cells/well in 96-well plates and incubated with proteasome inhibitors or drug solvent for 6 h, at various concentrations, followed by an additional 30-min incubation in reaction buffer (30 mM Tris–HCl pH7.5, 1 mM ATP, 5 mM MgCl2, 5 mM NaF, 1 mM DTT, and 100 μM Na_3VO_4) containing 0.5% NP40 and either Z-Leu-Leu-Glu-AMC (caspase-like), or succinyl-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like) at 100 μM. After addition of 200 μl cold ethanol, fluorescence of released aminomethylcoumarin within cells was measured with a spectrofluorimeter, as reported above. Trypsin-like activity was not evaluated in this type of experiment because in cells non-specific cleavage of the fluorogenic substrate methoxysuccinyl-Succ-Leu-Leu-Arg-aminomethylcoumarin used to measure trypsin-like activity was observed.

2.9. NF-κB activation assay

The 293T-NF-κB cell line, transfected with a plasmid containing the firefly luciferase gene to report for NF-κB activation, was used to determine the effects of physalin B or bortezomib on TNFα-induced NF-κB activation (Panomics, CA, USA). 293T-

NF-κB cells were preincubated with physalin B, bortezomib or drug solvent for 30 min at the indicated concentrations before stimulation by 50 ng/ml TNFα for 6 h. Luciferase activity was then measured in cell lysates using a LB 960 Centro luminometer (Berthold, France) following the instructions of a luciferase assay kit (Promega, France).

2.10. Apoptosis assay

DLD-1 4Ub-Luc cells were seeded at 10^4 cells/chamber onto chamber slides (Lab-Tek chamber slides treated for cell culture, Falcon, USA) and after 48 h they were incubated with physalin B, camptothecin (positive control) or drug solvent for 48 h at indicated concentrations, followed by an additional incubation with DEVD-MR (Ac-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-Magic Red) for 20 min and then Hoechst stain for 5 min, according to the manufacturer instructions (MP Biomedicals, Enzyme System Products, CA, USA). Apoptosis is detected via the enzymatic hydrolysis by caspase 3/7 of its fluorogenic DEVD-MR substrate, which produces a red fluorescence within cells. Cells were then viewed under a fluorescence microscope using a band-pass filter to detect either Magic Red or Hoechst (blue) fluorescence.

2.11. Cell proliferation assay

Physalin-induced cell proliferation inhibition was evaluated against A549, BxPC3, LoVo, Namalwa or SKOV3 cells, using the ATPlite kit assay, based on the measurement of ATP released from viable cells (Luminescence ATP detection, PerkinElmer, Ireland). After seeding in 96-well plates, cells were allowed to reach logarithm growth (24 h) before adding physalin B or solvent (0.1% DMSO) at selected concentrations for indicated times. Three independent experiments were performed and results were expressed as IC_{50} values (concentration of test compound that inhibits 50% of cell proliferation) from pooled data.

3. Results

3.1. DLD-1 4Ub-Luc, an engineered cell line to identify novel inhibitors of the ubiquitin-proteasome pathway

To directly assay 26S proteasome activity or inhibition in living cells, a ubiquitin-luciferase reporter was engineered in the human DLD-1 colon cancer cells (DLD-1 4Ub-Luc). Firefly luciferase was fused to four copies of a mutant ubiquitin (ubiquitin^{G76V}) that cannot be cleaved by ubiquitin hydrolases. As previously reported, once the ubiquitin chains reach a critical length of four or more ubiquitin moieties, the ubiquitinated protein is recognized with high affinity by the proteasome and degraded [17]. As a control, wild-type firefly luciferase was also stably transfected in DLD-1 cells (DLD-1 Luc). As expected, fusion of four ubiquitins destabilizes firefly luciferase. Indeed, whereas unfused luciferase remained stable in DLD-1 cells for more than 4 h, the fusion protein 4Ub-Luc half-life was only of 30 min, and upon treatment with a proteasome inhibitor, namely bortezomib, 4Ub-Luc half-life was similar to that of wild-type luciferase [11]. This suggests that 4Ub-Luc is efficiently degraded by proteasome. Furthermore, based on the studies of Zhu et al. [18] and Stack et al. [10], we made the hypothesis that the 4Ub-Luc reporter protein is polyubiquitinated in DLD-1 4Ub-Luc. Our experimental data supported this hypothesis. More specifically, after an 8 h-exposure of DLD-1 4Ub-Luc cells with 0.1 μM epoxomicin, the expressed 4Ub-Luc fusion proteins were immunoprecipitated with anti-luciferase antibody, recovered and separated by SDS-PAGE, followed by immunoblotting. The results revealed the presence in the precipitate of a smear of higher molecular weight proteins recognized by both anti-luciferase and anti-ubiquitin antibodies in addition to the expected 94 kDa 4Ub-Luc band. This high molecular weight protein smear was absent from immunoprecipitate performed in the same conditions from wild type luciferase expressing cells DLD-1 Luc (data not shown).

To determine whether 4Ub-Luc assay could detect differences in proteasome activity, DLD-1 4Ub-Luc cells were treated with increasing concentrations of proteasome inhibitors (Fig. 1). We determined that a statistically significant increase of bioluminescence is reflected by an Induction Factor $\geq$10. Each compound induced a dose-dependent increase in bioluminescence from DLD-1 4Ub-Luc cells, whereas they did not modify bioluminescence from DLD-1 Luc cells (Fig. 1A). Bortezomib appeared to be the most potent compound producing a 34-fold increase in bioluminescence at 0.01 μM, with a maximal value of 83-fold at 0.1 μM. Epoxomicin and MG-262 also induced a strong increase in bioluminescence from DLD-1 4Ub-Luc cells, as reflected by maximal increases of 83-fold and 80-fold, respectively, whereas lactacystin produced a lower increase in bioluminescence of 40-fold at 1 μM. To test the specificity of the DLD-1 4Ub-Luc assay to report for proteasome inhibition, the effects of reference anticancer agents that do not inhibit proteasome functions were examined. For example, etoposide, a topoisomerase II inhibitor and monastrol, a kinesin EG5 inhibitor did not increase the bioluminescence from DLD-1 4Ub-Luc cells at concentrations up to 10 μM (Fig. 1B). Other established anticancer drugs, such as camptothecin and various *Vinca* alkaloids, have been tested but none of them affected the bioluminescence from DLD-1 4Ub-Luc cells (data not shown). These results strongly suggest that our assay based on the use of DLD-1 4Ub-Luc specifically reports proteasome activity in cultured cells. It represents a robust tool for screening novel proteasome inhibitors.

3.2. Physalin B showed proteasome inhibitory properties

The DLD-1 4Ub-Luc assay was adapted to a high throughput screening platform [11]. Initially, over 30,000 compounds from plant extract collections and chemical libraries were screened, which led to several hits amongst which physalin B (Fig. 2A) was identified from a methanol extract of *P. angulata* aerial parts [11]. The activity of physalin B was then confirmed using the non-automated assay. As illustrated in Fig. 2B, physalin B induced a concentration- and time-dependent increase in bioluminescence from DLD-1 4Ub-Luc cells, reflecting its effect of stabilization of the 4Ub-Luc reporter protein in these cells and therefore the inhibition of 4Ub-Luc degradation by the proteasome. A significant increase in bioluminescence

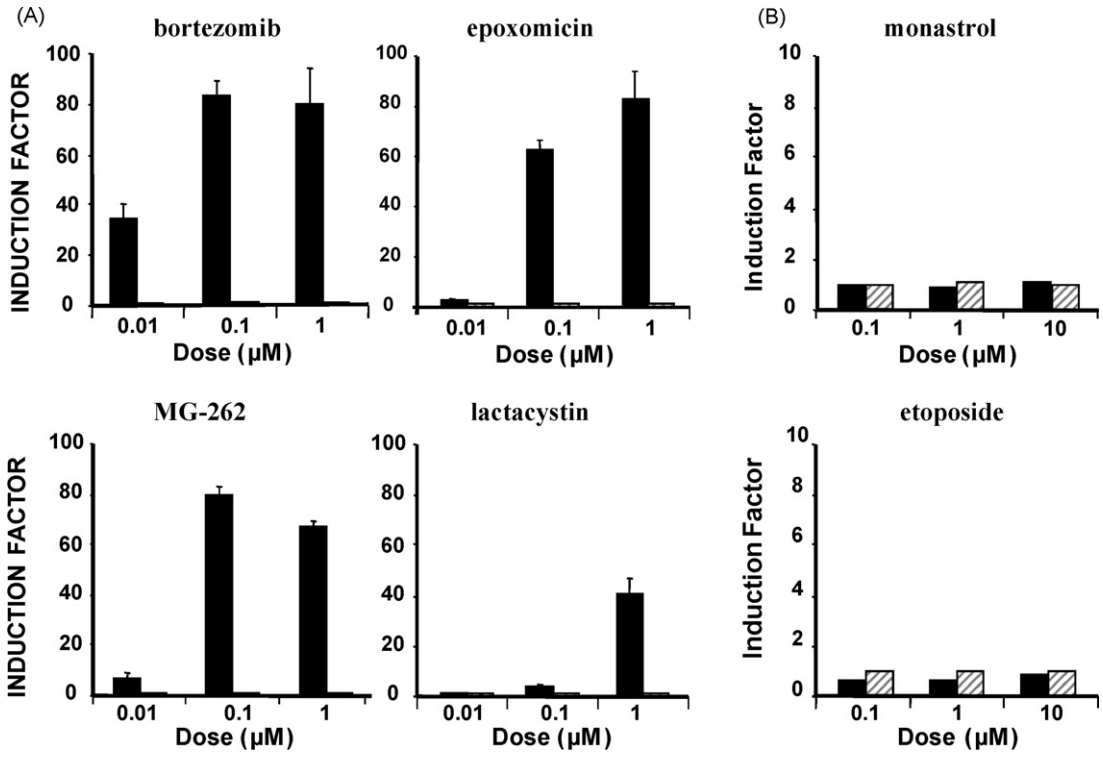

Fig. 1 – Inhibition of the proteasome produces concentration-dependent increases in bioluminescence from 4Ub-Luc. Stably transfected DLD-1 4Ub-Luc (filled bars) and DLD-1 Luc cells (hatched bars) were treated with increasing concentrations of: (A) bortezomib, epoxomicin, MG262 or lactacystin, or: (B) monastrol or etoposide for 8 h. Luciferase activity was determined in cell lysates. Increase in bioluminescence is expressed as an Induction Factor, defined as: RLU measured in presence of inhibitor/RLU measured in absence of inhibitor, with RLU being the relative light unit. Data, representative of three independent experiments, are expressed as a mean with the associate error of the estimate of the mean value.

was already observed after 6 h, with an Induction Factor of 17-fold at 5 μM. The maximal activity was obtained at 5 μM and after 16 h with a 33-fold increase in bioluminescence (Fig. 2B). The increase in bioluminescence was less important at 10 μM, which might result from a cytotoxic effect. Consistently with physalin B-induced increase in bioluminescence, ubiquitinated proteins were accumulated in DLD-1 4Ub-Luc cells treated with physalin B in a time- and concentration-dependent manner (Fig. 3A and B). A high level of protein accumulation was observed at 5 μM from 8 h and remained high until 48 h (Fig. 3A and B). More specifically, treatment of DLD-1 4Ub-Luc cells with 5 μM physalin B for 16 h induced accumulation of the cdk inhibitor p27, one of the well-known substrate of ubiquitin-proteasome pathway (Fig. 3C). Such effects were consistent with the biological effects judged as representative of proteasome inhibition [7]. Furthermore, to exclude the possibility that physalin B-induced inhibition of ubiquitin-proteasome pathway was due to a decreased level of ATP in DLD-1 4Ub cells, we assessed the effects of physalin B on the level of ATP, at times where inhibition of the ubiquitin-proteasome pathway was noted. Using an ATPlite kit assay, based on the measurement of ATP released from viable cells, we observed that physalin B at 5 μM for 6, 8 or 16 h did not modify the level of ATP in DLD-1 4Ub cells (data not shown). This suggests therefore that the inhibition of the degradation of 4Ub-Luc reporter protein and ubiquitinated proteins induced by physalin B after 6-16 h cannot be due to a leakage of ATP. Then to determine whether physalin B inhibits ubiquitin-proteasome pathway through inhibition of catalytic activities of proteasome, its effects on the chymotrypsin-like, trypsin-like and caspase-like activities of the purified proteasome were examined. Physalin B at concentrations up to 100 μM did not interfere with these enzymatic activities (Table 1A). In contrast, bortezomib, epoxomicin or clasto-lactacystin (lactacystin active metabolite) inhibited chymotrypsin-like activity with IC_{50} values of 0.02 μM, 0.09 μM, and 0.33 μM, respectively (Table 1A). To use a more sensitive assay, cellular proteasomal chymotrypsin-like and caspase-like activities were measured after treatment of DLD-1 4Ub-Luc cells with physalin B. We found that physalin B inhibited the cellular proteasomal chymotrypsin-like and caspase-like activities in DLD-1 4Ub-Luc cells but at 20 μM and 40 μM, respectively, which are approximately 4- to 8-fold higher concentrations than that required to induce significant increase in bioluminescence and accumulation of ubiquitinated proteins in DLD-1 4Ub-Luc cells (Table 1B, Figs. 2 and 3). In sharp contrast, bortezomib, epoxomicin and lactacystin inhibited cellular proteasomal chymotrypsin-like and caspase-like activities at 100-fold lower concentrations than those required to produce an increase in bioluminescence or accumulation of ubiquitinated proteins in DLD-1 4Ub-Luc cells (Table 1B and Fig. 3). Overall, these results indicate that

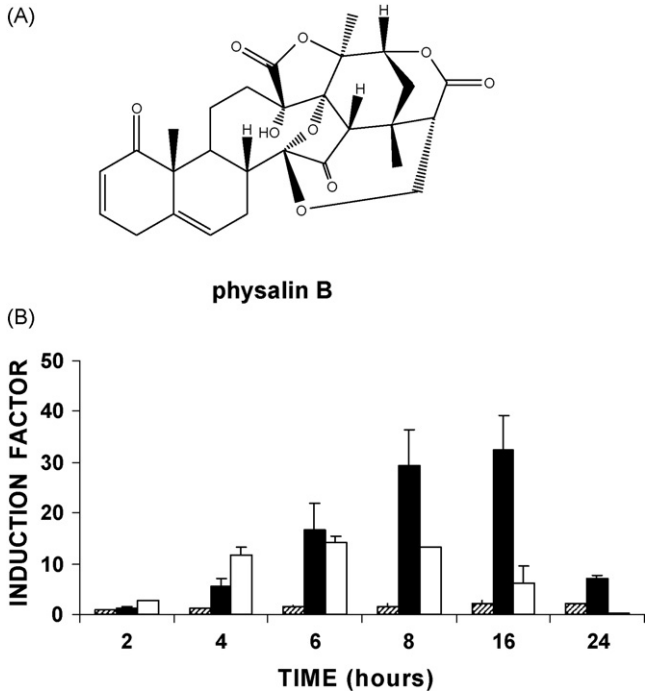

Fig. 2 – Physalin B induces an increase in bioluminescence from DLD-1 4Ub-Luc cells. (A) Chemical structure of physalin B. (B) Stably transfected DLD-1 4Ub-Luc were treated with 1 μM (hatched bars), 5 μM (black bars) or 10 μM (white bars) physalin B for 1–24 h. Luciferase activity was determined in cell lysates. Increase in bioluminescence is expressed as an Induction Factor, defined as: RLU measured in presence of inhibitor/RLU measured in absence of inhibitor, with RLU being the relative light unit. Data, representative of two independent experiments, are expressed as a mean with the associate error of the estimate of the mean value.

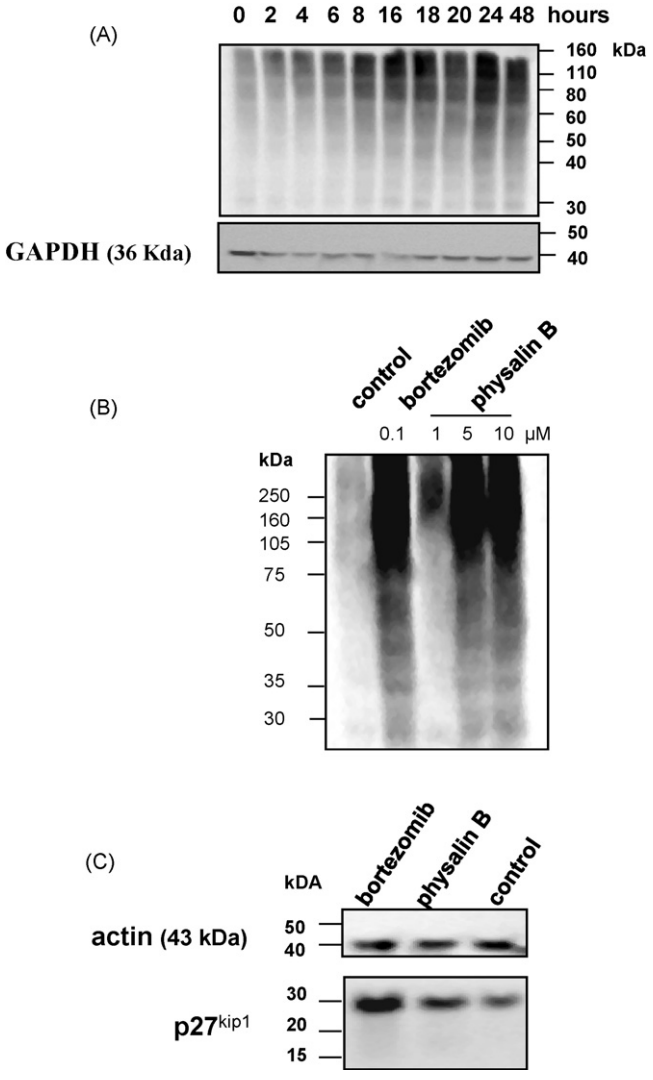

Fig. 3 – Physalin B induces accumulation of ubiquitinated protein and $p27^{kip1}$ in DLD-1 4Ub-Luc cells. Western blot analyses of the level of ubiquitinated proteins in DLD-1 4Ub-Luc cells after treatment with 5 μM physalin B at indicated times (A), or after a 16 h treatment with drug solvent (control), 0.1 μM bortezomib or the indicated concentrations of physalin B (B), or Western blot analyses of the level of $p27^{kip1}$ in DLD-1 4Ub-Luc cells after a 16 h treatment with drug solvent (control), 0.1 μM bortezomib or 5 μM physalin B (C).

physalin B is an inhibitor of the ubiquitin-proteasome pathway. Furthermore, they suggest that inhibition of the catalytic activities of proteasome might not be the only mechanism by which physalin B interferes with the ubiquitin-proteasome pathway.

3.3. *Physalin B inhibited TNFα-induced NF-κB activation*

NF-κB, a key transcription factor, is regulated primarily through interactions with an inhibitor protein known as IκB. This inhibitor is phosphorylated which leads to its ubiquitination and its subsequent degradation via the proteasome. After IκB degradation, NF-κB translocates to the nucleus, where it regulates various genes. Proteasome inhibitors have proved to block NF-κB activation through the inhibition of IκB degradation [5]. This prompts us to investigate the effects of physalin B on NF-κB activation. Physalin B inhibited TNFα-induced NF-κB activation in 293T-NF-κB cells, which express a luciferase reporter of NK-κB activation, in a concentration-dependent manner, with 29% inhibition at 2.5 μM and a maximal inhibition of 85%, reached at 5 μM (Fig. 4).

3.4. *Physalin B induced NOXA proapoptotic protein accumulation in DLD-1 4Ub-Luc cells*

It has been shown that proteasome inhibition is associated with the induction of NOXA, a proapoptotic member of the BH3-only family [19]. By analogy, the effect of physalin B on NOXA accumulation at the protein level was examined in DLD-1 4Ub-Luc cells, by Western blots (Fig. 5). Treatment of these cells with 5 μM physalin B resulted in a time-dependent increase of the level of NOXA, as compared to untreated cells (Fig. 5). NOXA accumulation was detected from 6 h and reached a maximal level at 16 h. Bortezomib, included as

Table 1 – Effects of physalin B on catalytic activities of purified or cellular proteasome

(A) Effects of physalin B on the catalytic activities of purified proteasome

Compound	CT-L IC_{50} (μM)	Caspase-L IC_{50} (μM)	T-L IC_{50} (μM)
Physalin B	>100	>100	>100
Bortezomib	0.02	0.82	2.60
Epoxomicin	0.09	>100	1.00
Clasto-lactacystin β lactone	0.33	>100	7.20

(B) Effects of physalin B on the proteasome catalytic activities in DLD-1 4Ub-Luc cells

Compound	CT-L IC_{50} (μM)	Caspase-L IC_{50} (μM)
Physalin B	20	40
Bortezomib	0.0002	0.0009
Epoxomicin	0.006	0.06
Lactacystin	0.07	0.30

Levels of chymotrypsin-like (CT-L), caspase-like (Caspase-L) or trypsin-like (T-L) activities of purified (A) or cellular (B) (DLD-1 4Ub-Luc cells) proteasome were measured using fluorogenic substrates specific of each activity. Data are expressed as IC_{50} values that are the concentration of test compound which inhibits 50% of the proteasome catalytic activity. Clasto-lactacystin β lactone is the active form (metabolite) of lactacystin required in a cell-free assay.

reference proteasome inhibitor, also induced NOXA accumulation in DLD-1 4Ub-Luc cells at 0.1 μM after 16 h (Fig. 5). In contrast, doxorubicin, an anticancer agent that does not interfere with proteasome activity, did not modify the level of NOXA (data not shown).

3.5. *Physalin B induced apoptosis in DLD-1 4Ub-Luc cells*

To investigate whether physalin B-induced NOXA accumulation is followed by apoptosis, levels of caspase-3/7 activity, PARP cleavage, as well as cellular morphological changes were examined in DLD-1 4Ub-Luc cells treated with physalin B (Fig. 6). A time-dependent cleavage of PARP was observed, with up to 100% PARP cleavage product being recorded after a 48-h exposure to 5 μM physalin B, and a partial cleavage detected after 24 h (Fig. 6A). Physalin B at 1 and 5 μM also induced caspases 3/7 activity after 48 h, as reflected by the red fluorescence produced by cleaved caspases 3/7 substrate within DLD-1 4Ub-Luc cells (Fig. 6B). As a positive control 20 μM camptothecin, a potent cytotoxic agent, known to trigger apoptosis, also induced caspases 3/7 activation in DLD-1 4Ub-Luc cells, whereas no red fluorescence was detected in cells treated with drug solvent. Furthermore, the blue staining of nuclei with Hoechst allowed to observe morphologic changes characteristic of apoptosis: chromatin condensation and fragmentation in physalin B-treated cells (Fig. 6B).

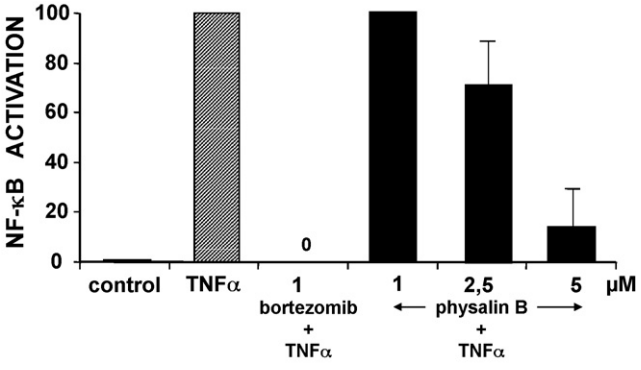

Fig. 4 – Physalin B inhibits TNFα-induced NF-κB activation. 293T-NF-κB cells were preincubated with either the drug solvent, bortezomib or the indicated concentrations of physalin B for 30 min before addition of 50 ng/ml TNFα for 6 h. Luciferase activity was measured in cell lysates and data are expressed as Induction Factors, relative to control (drug solvent treatment). Induction factors are then expressed in values relative to TNFα-induced NF-κB activation. Results from two independent experiments are means with the associate error of the estimate of the mean value.

3.6. *Inhibition of cell proliferation*

The capacity of physalin B to inhibit cell proliferation *in vitro* was determined using a panel of human tumor cell lines from various histological origins, namely lung (A549), pancreas (BxPC3), lymph (Namalwa) and ovary (SKOV3) and also DLD-1 4Ub-Luc. A significant suppression of cell growth was detected in the presence of physalin B, with IC_{50} values of 2 μM for A549, BxPC3, Namalwa, 3 μM for SKOV3 and 1 μM for DLD-1 4Ub-Luc, after 72 h of drug treatment.

4. Discussion

The remarkable success of proteasome inhibitors in the treatment of cancer, inflammatory disorders and stroke in animal models and clinical trials encourage researchers to identify novel, second generation agents. This study reports that the DLD-1 4Ub-Luc cell line, reporter of proteasome activity or inhibition, provides an efficient tool to identify novel inhibitors of the ubiquitin-proteasome pathway. Screening of plant collections led to the identification of physalin B from *P. angulata*, which demonstrated proteasome inhibitory properties associated with the inhibition of TNFα-induced NFκB activation and the induction of the proapoptotic NOXA protein.

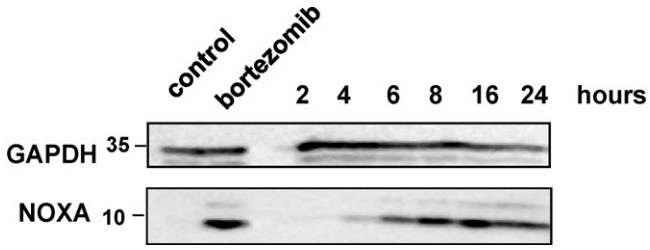

Fig. 5 – Physalin B induces an increase in the level of the NOXA proapoptotic protein in DLD-1 4Ub-Luc cells. Western blot analyses of the level of NOXA in DLD-1 4Ub-Luc cells after treatment with 5 μM physalin B at indicated times, or with 0.1 μM bortezomib for 16 h.

This study further reports that physalin B induced apoptosis in DLD-1 4Ub-Luc cells through PARP cleavage and caspases 3/7 activation and exhibited cytotoxicity against a panel of human tumor cell lines.

The search for novel anticancer agents from natural sources is still an important approach for cancer prevention and therapy. Various proteasome inhibitors were isolated from natural resources. Lactacystin or epoxomicin were isolated from Streptomyces lactacystinaeus and an Actinomycetes strain, respectively [20]. Salinosporamide A (NPI-0052), recently characterized from the marine Gram-positive actinomycete Salinospora tropica is a promising proteasome inhibitor with potent anticancer properties [21]. Various other compounds isolated from fermentation broth of microorganisms, like gliotoxin, belactosin, TMC-95A or tyroptin A proved to interfere with proteasome function through inhibition of chymotrypsin-like activity [20]. Furthermore, amongst the inhibitors of the other steps of the ubiquitin-proteasome pathway, panepophenanthrin from a mushroom strain, Panus rudis and himeic acid A from a culture of marine-derived fungus (Aspergillus) were identified as inhibitors of the ubiquitin-activating enzyme E1 and chlorofusin from the culture of a Fusarium strain showed to be an inhibitor of the MDM2 ubiquitin ligase E3 [22–24]. This illustrates the diversity of natural compounds interfering with the ubiquitin-proteasome pathway. Consistently with this context, we identified physalin B from aerial parts of the plant *P. angulata* as an inhibitor of the ubiquitin-proteasome pathway, using the DLD-1 4Ub-Luc assay, reporter of proteasome activity. The use of a cellular assay as a primary screening allows us to demonstrate at the first step that an inhibitor is able to penetrate cells. This is not the case for most of the compounds described above since they were mainly screened for their capacity to inhibit the activities of purified enzymes.

To the best of our knowledge, the proteasome inhibitory properties of physalins have not been reported by other groups. However, Jacobo-Herrera et al. [25] recently showed that physalins B and D inhibited PMA-induced NF-κB activation at 16 and 8 μM, respectively. These data support our findings showing that physalin B inhibited TNFα-induced NF-κB activation at 5 μM. Furthermore, physalin B induced the accumulation of the 4Ub-Luc reporter protein in DLD-1 4Ub-Luc cells at 5 μM from 6–8 h, which is also a concentration and a time at which the inhibition of ubiquitinated protein degradation by proteasome, and more specifically p27 were observed in DLD-1 4Ub cells. These findings are consistent with the biological effects judged as representative of proteasome inhibition [7] and therefore support the conclusion that physalin B interferes with the ubiquitin-proteasome pathway. However, physalin B appears to be a weak inhibitor of proteasome catalytic activities. Indeed, it did not inhibit chymotrypsin-like, tryspsin-like or caspase-like activities of purified proteasome, whereas bortezomib, epoxomicin or lactacystin interfered potently with these enzymatic activities. Using a more sensitive assay, we showed that physalin B inhibited cellular proteasomal

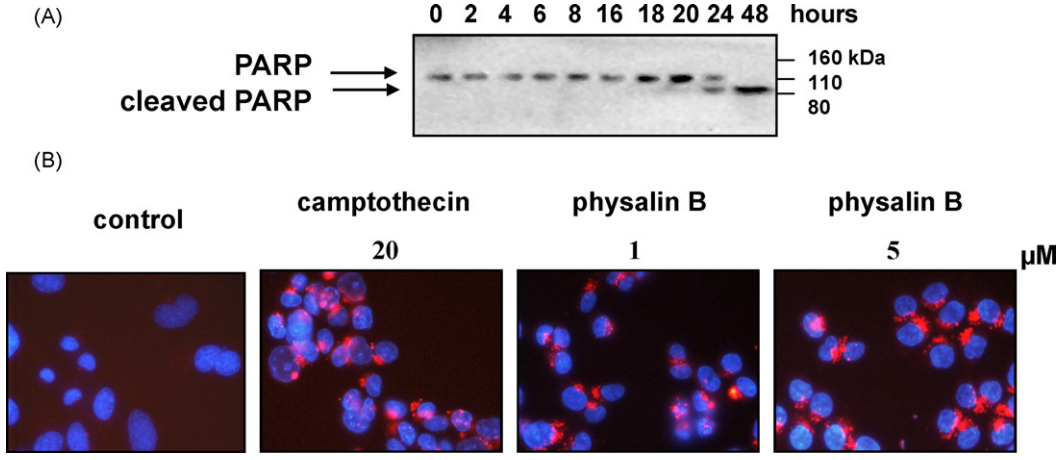

Fig. 6 – Physalin B induces apoptosis in DLD-1 4Ub-Luc cells. (A) Western blot analyses of the level of PARP (113 kDa) and cleaved form of PARP (89 kDa) in DLD-1 4Ub-Luc cells after treatment with 5 μM physalin B at indicated times (B) DLD-1 4Ub-Luc cells were incubated with either drug solvent, or a compound included as a positive control, namely camptothecin, or the indicated concentrations of physalin B for 48 h, followed by an additional incubation with DEVD-MR, a caspase 3/7 substrate for 20 min and then Hoechst stain for 5 min. Cells were then viewed under a fluorescence microscope using a band-pass filter to detect either Magic Red or Hoechst (blue) fluorescence, with a ×40 objective. Pictures were then superimposed.

chymotrypsin-like and caspase-like activities at 20 and 40 μM, respectively. However, these concentrations are 4- to 8-fold higher than that inducing the inhibition of the ubiquitin-proteasome pathway, *i.e.*, 5 μM. In sharp contrast, bortezomib, epoxomicin or lactacystin inhibited cellular proteasomal chymotrypsin-like and caspase-like activities at 100-fold lower concentrations than those required to produce an increase in bioluminescence or accumulation of ubiquitinated proteins in DLD-1 4Ub-Luc cells. This suggests that the mechanisms by which physalin B interferes with proteasome functions might be different from those of bortezomib, epoxomicin or lactacystin. Physalin B might also interfere with steps upstream of proteolysis. Indeed, the 4Ub-Luc reporter assay should allow to identify compounds interfering with multiple steps of the ubiquitin-proteasome pathway including, ligation of additional ubiquitin molecules to the 4Ub-Luc reporter protein, ubiquitinated protein binding to 19S regulatory particle, ubiquitin chain removing, opening of the gate and translocation into the catalytic chamber of the 20S core particle and proteolysis. These steps upstream of proteolysis involve many regulatory particles that constitute the base of the 19S part which directly interacts with the α face of the 20S core, such as Rpt1-6 (Regulatory particles ATPase) with ATPase activity, and non-ATPase subunits, like Rpn (Regulatory particles non-ATPase) [26]. The functions of these regulatory particles might potentially be modified by physalin B. Ubistatins are an example of compounds interfering with proteasome-dependent degradation without inhibiting catalytic activities of proteasome, but by binding the ubiquitin chain of ubiquitinated substrates, preventing therefore their binding to the proteasome [27]. This compound acts by disrupting a critical protein-protein interaction in the ubiquitin-proteasome pathway. We can also make the hypothesis that physalin B binds the proteasome to a site different from the catalytic site, thereby bringing about a conformational change such as to alter the catalytic activity or preventing access to the catalytic chamber of protein that has to be degraded. Therefore interfering indirectly with the catalytic site, a high concentration of physalin B would be necessary to alter its activity. In that case, physalin B would act as a noncompetitive or allosteric inhibitor of proteasome. As suggested by Tan et al. [26], the proteasome, with its multiple subunits, activities and regulatory proteins, is an ideal candidate to be an allosteric structure.

It has been shown that proteasome inhibitors, including bortezomib, epoxomicin and MG-132 triggered NOXA-mediated apoptosis in several cancer cell lines [19,28]. Furthermore, based upon the findings that this proapoptotic protein NOXA was induced by bortezomib in melanoma cells but not in normal melanocytes, it has been proposed that NOXA could be a biomarker of the efficacy of proteasome inhibitors specifically in tumor cells [29]. Therefore, having identified a novel proteasome inhibitor, we investigated the effects of physalin B on NOXA induction and found that physalin B also induced accumulation of the NOXA protein in DLD-1 4Ub-Luc cells, at concentrations and times that caused proteasome inhibition. Physalin B, at the same dose level, also triggered apoptosis in DLD-1 4Ub-Luc cells, as reflected by caspases 3/7 activation, PARP cleavage and morphological changes. However, physalin B-induced cell death was observed at 24–48 h, thus following the events reflecting proteasome inhibition, namely, 4Ub-Luc reporter protein accumulation and ubiquitinated protein degradation inhibition, that were detected as early as after a 6–8 h-exposure to physalin B. Therefore, these data suggest that physalin B-induced proteasome inhibition has triggered apoptosis.

Our data also show that physalin B displayed cytotoxicity against a panel of human tumor cell lines, with IC_{50} values in the micromolar range. Many reports have discussed the anticancer potential of *P. angulata* and its constituents [30–33]. For example, Ferreira Magalhaes et al. [34], reported that physalins B and D exhibited cytotoxicity against several cancer cell lines with IC_{50} ranging from 1 to 30 μM. *In vivo* antitumor activity of physalin B was also demonstrated using the murine sarcoma 180 or 3PS leukemia models [34,12]. But these previous studies provided no information about the potential mechanism of action of physalin B, which we have now characterized, at least partially.

In conclusion, this report indicates that the DLD-1 4Ub-Luc assay, reporter of proteasome activity in cultured cells, is an efficient screening tool for discovery of novel inhibitors of the ubiquitin-proteasome pathway. Thanks to this assay, the proteasome inhibitory properties of physalin B were identified. These findings were further confirmed by ubiquitinated protein accumulation and the inhibition of TNFα-induction NFκB activation. Furthermore, our data suggest that the mechanism by which physalin B interferes with proteasome functions might be different from those of reference proteasome inhibitors, namely bortezomib or epoxomicin or lactacystin. Physalin B also induced an increase of the level of the proapoptotic protein NOXA, identified as a component of the overall cell killing mechanisms of proteasome inhibitors. Consistently, physalin B triggered apoptosis and displayed cytotoxic properties following proteasome inhibition. This confirms and extends previous studies suggesting that physalin B exhibits anticancer properties. The remaining challenge is to specify the mechanism by which physalin B interferes with ubiquitin-proteasome pathway and to further use physalin B or design, synthesize and evaluate more potent and selective physalin B analogs with toxicity and activity profiles compatible with a clinical use.

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