Identification of an inhibitor of the ubiquitin–proteasome system that induces accumulation of polyubiquitinated proteins in the absence of blocking of proteasome function

Caroline Haglund,⁎a Chitralekha Mohanty,⁎b Mårten Fryknäs,a Padraig D’Arcy,b Rolf Larsson,a Stig Linder⁎ab and Linda Rickardsona

The ubiquitin–proteasome system (UPS) represents one of the most promising therapeutic targets in oncology to emerge in recent years. Here we used a combination of cytotoxic and image-based screening assays to identify a novel UPS inhibitor, designated HRF-3. HRF-3 evokes a gene expression profile similar to that of other characterized UPS inhibitors, suggesting a common mechanism of action. Consistent with UPS inhibition, HRF-3 induced strong accumulation of polyubiquitinated proteins in cells. Surprisingly, HRF-3 induced only weak accumulation of two proteasome targeted reporter proteins, UbG76V-YFP and ZsGreen-ODC. Consistent with this observation, HRF-3 did not inhibit proteasome proteolytic activity in an in vitro assay. Similar to a number of other UPS inhibitors, HRF-3 increased the expression of the redox-inducible protein Hmox-1. In distinction to the 20S inhibitor bortezomib, but similarly to two different p97/VCP inhibitors, HRF-3 did not elicit strong induction of the chaperone Hsp70B. Finally, we show that HRF-3 is cytotoxic to a variety of cancer cell lines and ex vivo patient tumour cells, with the strongest activity observed in cells of leukemic/myeloma origin. Taken together our data show that HRF-3 induces polyubiquitin accumulation in the absence of efficient proteasomal blocking, and suggest that induction of oxidative stress is a common denominator of UPS inhibitors.

Introduction

The ubiquitin–proteasome system (UPS) is the main pathway for protein degradation in eukaryotic cells. Ubiquitinated proteins are degraded by the 26S proteasome, a large proteolytic complex composed of a 20S enzymatic core capped by one or two regulatory 19S complexes. The 20S core particle is a cylinder-shaped complex composed of 4 stacked heptameric α- and β-subunits that harbor three distinct enzyme-like activities, chymotryptic-like, tryptic-like and post-glutamyl-peptidyl-hydrolytic-like activity, which hydrolyze target proteins based on their respective substrate preferences.¹

The targeting of proteins to the proteasome for degradation is initiated by the covalent attachment of poly-ubiquitin chains, which serve as highly specific destruction signals.³ Whereas the 20S proteasome functions as the molecular shredder, the 19S complex functions as a selective gate keeper, only allowing ubiquitin-tagged proteins access to the 20S proteasome for degradation. The attachment of ubiquitin is selective and requires a sequential action of E1, E2 and E3 enzymes that activate the ubiquitin molecule and catalyse its covalent attachment to target proteins. This process is highly dynamic and can be reversed by specific deubiquitination enzymes (DUBs) adding an additional mechanism of regulation. It has been suggested that several enzymes of the ubiquitination cascade are differently expressed or activated in cancer and may therefore be appropriate drug targets.⁴,⁵

Malignant cells synthesize large quantities of proteins and thus require a functioning system for degrading misfolded proteins that would otherwise accumulate and result in proteotoxicity. Consistent with this, tumour cells have been shown to be more susceptible to proteasome inhibition compared to normal cells.¹ The approval of the proteasome inhibitor bortezomib (Velcade®) for treatment of multiple myeloma and mantle cell lymphoma has verified this concept. Bortezomib displays potent anti-tumour activity and is generally well-tolerated,⁶,⁷ however several problems have emerged including dose-limited toxicity and acquired drug resistance which occurs in the majority of patients, suggesting a need to identify new inhibitors of the UPS.
The UPS is believed to be highly druggable, but has been underexplored by drug developers. A number of structurally unrelated molecules have been shown to inhibit the proteasome (reviewed in ref. 9). Interestingly, several cytotoxic compounds such as piperlongumine,18,19 siomycin A20 and WP1130,4 initially described to have alternative mechanisms of action, were subsequently shown to be potent inhibitors of the UPS. Some compounds, such as physalins A21 and piperlongumine,18 were reported to inhibit the UPS at a level distinct from the 20S proteasome, but the exact mechanisms of action have not been characterized.

Considering both the structural diversity of proteasome inhibitors and the subsequent reclassification of previously unknown proteasome inhibitors, we were interested in determining the prevalence of UPS inhibitors amongst panels of cytotoxic drugs. From an initial library of 10 000 drug-like small molecules we identified 382 as having anti-proliferative activity. Using an image-based assay based on proteasome inhibition we identified one compound as a potential inhibitor of the UPS. This compound named HRF-3 showed interesting anti-neoplastic properties, which we describe here.

Results

Identification of a novel UPS inhibitor

Proteasome inhibition was determined using an image-based method to monitor the accumulation of an ornithine decarboxylase-green fluorescent fusion protein (ZsGreen-ODC) in HEK 293 cells. This fusion protein is unstable in cells due to rapid degradation by the proteasome.14 From an initial screen of 382 compounds that displayed promising cytotoxic properties, a single compound was identified to induce accumulation of the reporter protein at a level >3 S.D. above that of untreated control cells (Fig. 1A). The structure of this compound (phosphoric acid, 2,3-dihydro-1,1-dioxido-3-thienyl diphenyl ester), here designated HRF-3, is shown in Fig. 1B. To validate the result we examined the ability of HRF-3 to block the degradation of another proteasome substrate (UbG76V-YFP), expressed by MelJuSo melanoma cells. HRF-3 was found to induce the accumulation of UbG76V-YFP also (Fig. 1C). The fluorescent signals obtained in both of these assays were weak compared to those obtained following treatment with the 20S proteasome inhibitor bortezomib (Fig. 1A and C).

The gene expression response to HRF-3 is similar to that of known UPS inhibitors

To characterize the cellular response to HRF-3, we took advantage of the Connectivity Map database (Cmap),15 a compendium of gene expression signatures generated from drug-exposed human tumour cell lines. The Cmap database contains gene expression profiles for 1309 compounds, including the gene expression pattern induced by HRF-3 (ChemBridge ID 5155877). The gene expression profile generated by HRF-3 treatment showed considerable similarity to the profile of the well characterized proteasome inhibitor MG-262, as well as those of thiostrepton,12 15β-PGJ216 and withaferin A17 compounds that have been shown to act as inhibitors of the UPS (Fig. 2). We conclude that HRF-3 induces a gene expression profile similar to that observed with several UPS inhibitors.

HRF-3 induces accumulation of polyubiquitinated proteins but does not block proteasome function in cells

Inhibition of the UPS typically results in increased levels of high molecular weight polyubiquitinated proteasomal substrates in cells. We indeed found an accumulation of K48-linked polyubiquitinated proteins over time in MelJuSo cells following treatment with 5 μM HRF-3 (Fig. 3A). In both MelJuSo and HCT116 cells, the extent of polyubiquitin accumulation was similar to that observed using the 20S proteasome inhibitor bortezomib (Fig. 3B and C). Simultaneous exposure to HRF-3 and bortezomib did not result in further increases of polyubiquitinated proteins (Fig. 3C). The strong accumulation of polyubiquitin was surprising considering the weak signals from the proteasome substrates ZsGreen-ODC and UbG76V-YFP (Fig. 1A and C). We compared the accumulation of UbG76V-YFP in HRF-3- and bortezomib-exposed cells using western blotting. In the experiment shown in Fig. 3B, UbG76V-YFP could not be detected after exposure to 5 μM HRF-3 whereas the levels of the reporter were strongly increased by bortezomib.

To directly address whether HRF-3 inhibits the UPS at the level of the 20S proteasome, we investigated whether HRF-3 inhibits the enzymatic activity of the 20S proteasome using SucLLVY-AMC as a substrate. No inhibitory activity was observed at 10 μM and only weak inhibition was observed at 50 μM (Fig. 3D) whereas, as expected, the 20S inhibitor bortezomib completely inhibited enzymatic activity.

Weak induction of Hsp70B expression and strong induction of Hmx-1 expression by HRF-3

We further examined the relationship between accumulation of proteasome substrates and polyubiquitinated proteins. Similar levels of K48-linked polyubiquitinated proteins were detected in cells exposed to between 5 and 100 μM HRF-3 (Fig. 4A). In contrast, UbG76V-YFP was detected by immunoblotting at an HRF-3 concentration of 50 μM (~10-fold higher than the IC50 concentration) (Fig. 4A). An increase of the CDK-inhibitor p21, a known proteasome substrate,18 was observed at 20 μM with stronger increase at 50 μM.

Chaperone induction is expected under conditions of accumulation of misfolded proteins. HRF-3 indeed induced expression of chaperone genes such as HSPA1A (Hsp70-1A), HSPA1B (Hsp70-1B) and DNAJB4 (Hsp40) (Fig. 2). Interestingly, however, HSPA6 transcripts (encoding the chaperone Hsp70B) were not induced by HRF-3. Hsp70B is strongly induced by the proteasome inhibitor bortezomib19 and by the deubiquitinase inhibitor b-AP15,20 and is considered as a final cellular defence mechanism against proteotoxic stress.21 As shown in Fig. 4A, Hsp70B protein levels did not increase at HRF-3 concentrations of 5–20 μM, but were induced at the concentration where the proteasome was inhibited (50 μM).

We compared the response to HRF-3 to that of other compounds described to inhibit the UPS (Fig. 4B). NMS859 and...
eeyarestatin-1 (ES-1) are two recently described inhibitors of p97/VCP, an AAA(+) ATPase regulating endoplasmic reticulum-associated degradation. NMS859 inhibits the ATPase activity of this enzyme, whereas ES-1 inhibits p97/VCP-associated deubiquitinase activity. Piperlongumine was recently shown to inhibit the UPS independently of the activities of the 20S and 19S proteasome. All three compounds induced the accumulation of polyubiquitinated proteins in MelJuSo cells at concentrations of 5–10 μM (Fig. 4B) but caused little or no accumulation of the UbG76V-YFP reporter.

We also examined the induction of Hsp70B expression by these different UPS inhibitors and examined the expression of the redox-regulated marker Hmox-1. Similar to HRF-3, but distinct from bortezomib and b-AP15, p97/VCP inhibitors and piperlongumine did not induce strong Hsp70B expression (Fig. 4B). In contrast, all UPS inhibitors examined here elicited increases in Hmox-1 expression, the strongest increases observed with ES-1 and HRF-3 (Fig. 4B). HMOX-1 is a target gene of the redox-regulated nuclear factor-erythroid-2-related factor 2 (Nrf-2) and induction of this protein is consistent with previous reports that proteasome inhibitors induce oxidative stress.

We conclude that HRF-3 elicits a response characterized by accumulation of polyubiquitinated proteins and increased expression of Hmox-1, whereas Hsp70B was not strongly induced at cytotoxic concentrations.

**HRF-3 is cytotoxic to cancer cell lines and primary patient tumour cells**

Since HRF-3 inhibits the UPS by a mechanism distinct from that of the clinically used agent bortezomib, we were interested in the potential activity of the drug on tumour cells. The cytotoxic activity of HRF-3 was first investigated in eight cell lines (Table 1). Myeloma cell lines are known to be highly sensitive to proteasome inhibitors. Consistent with this, the myeloma cell lines NCI-H929 and RPMI 8226 showed the highest degrees of sensitivity, with IC_{50} values of 0.61 μM and 1.3 μM, respectively. The drug-resistant sub-line 8226/Dox40 and the leukemic cell lines CCRF-CEM, Kasumi-1 and HL-60 were slightly less sensitive and colon carcinoma HCT116 and MelJuSo melanoma cells were the most resistant. To investigate the anti-tumour activity of HRF-3 in a more clinically relevant setting, we treated a diverse set of primary tumour samples from patients representing colon carcinoma, pseudomyxoma peritonei (PMP), acute myeloid leukaemia (AML) and chronic lymphoblastic leukaemia (CLL) (Fig. 5). We included bortezomib as a reference UPS inhibitor. The two drugs displayed a similar pattern of...
activity on the tumour types tested. CLL cells displayed the highest degree of sensitivity to HRF-3 with a median IC$_{50}$ value of 0.69 $\mu$M, followed by colon carcinoma samples with a median IC$_{50}$ of 1.7 $\mu$M (Fig. 5A). The response of pseudomyxoma peritonei (PMP) was highly variable, with IC$_{50}$ values ranging from 0.51 $\mu$M to 30 $\mu$M. A similar pattern of sensitivity was observed to bortezomib, with CLL cells being the most sensitive followed by colon carcinoma (Fig. 5B). These data suggest that HRF-3 displays anti-tumour activity on a variety of cancer types.

Discussion

The gene expression profile induced by the small molecule HRF-3 was similar to that of compounds previously shown to act as UPS inhibitors, such as MG-262, withaferin A, thiostrepton and 15-delta PGJ2. As expected from UPS inhibition, we observed strong accumulation of polyubiquitinated proteins in tumour cells. Polyubiquitinated proteins generally accumulate as a result of decreased proteasome function, and are observed after treatment with inhibitors of 20S enzymatic activity or 19S deubiquitinase activity. Interestingly, at cytotoxic concentrations HRF-3 induced only weak accumulation of the ubiquitin-independent proteasome substrate ZsGreen-ODC and the ubiquitin-dependent proteasome substrate Ub676V-YFP. Induction was, however, sufficient to be detected in an image-based screening system. In immunoblotting experiments, increased levels of Ub676V-YFP were detected at an HRF-3 concentration of 50 $\mu$M, but not at IC$_{50}$ concentrations (Fig. 4A). These findings suggested that the proteasome is only partially inhibited by HRF-3 treatment at cytotoxic concentrations. This pattern is distinct from that observed with the 20S proteasome inhibitor bortezomib and the 19S proteasome deubiquitinase inhibitor b-AP15 which both induce strong accumulation of Ub676V-YFP in cells (Fig. 4B). For both these inhibitors, accumulation of Ub676V-YFP is strongly associated with cytotoxicity. These observations raise questions with regard to the origin of polyubiquitinated proteins observed in HRF-3-treated cells and why these proteins are not recognized and degraded by the proteasome.

Drug screening has identified other small molecules that inhibit the UPS at levels other than the proteasome. Piperlongumine is a natural product from the plant Piper longum (Long pepper) described to induce oxidative stress and to induce tumour cell-specific cell death. We and others recently reported that piperlongumine is a UPS inhibitor that generates polyubiquitinated proteins in the absence of 20S proteasome inhibition. We here confirmed the induction of polyubiquitinated proteins in the absence of accumulation of Ub676V-YFP by this compound. Physalin B is a steroid-like compound isolated from the plant Physalis angulata that was identified in a cell-based screen for UPS inhibitors similar to ours. Physalin B increases the levels of polyubiquitinated proteins but does not block the enzymatic activity of the 20S proteasome. HRF-3, piperlongumine and physalin B are likely to block the UPS at step(s) upstream of the proteasome, such as endoplasmic reticulum (ER)-associated protein degradation (ERAD), shuffling of polyubiquitinated substrate proteins to
The proteasome or binding of substrates to ubiquitin receptors. p97 (valosin-containing protein: p97/VCP; Cdc48p in yeast) is an AAA(+) ATPase that is instrumental for extraction of ERAD substrates. We here examined the effects of NMS859, an inhibitor of the p97/VCP ATPase, and eeyarestatin-1, an inhibitor of p97/VCP-associated DUB activity. We show that both inhibitors induce the accumulation of polyubiquitinated substrates in a manner similar to the proteasome inhibitor bortezomib, although neither compound induces accumulation of the UbG76V-YFP reporter. These results show that interference with the UPS at pre-proteasomal steps give a similar response to that observed with HRF-3, piperlongumine and physalin-B. The exact mechanism(s) of action of these different orphan UPS inhibitors will be interesting to evaluate in future studies.

Stabilization of various proteasomal substrates such as IkB and c-Myc have been suggested to being instrumental to proteasome inhibitor-induced cytotoxicity (reviewed in ref. 36 and 37). It is therefore interesting to note that HRF-3 as well as other UPS inhibitors such as piperlongumine, NMS859 and eeyarestatin-1 that do not appear to block proteasome degradative function are cytotoxic to tumour cells. Production of reactive oxygen species (ROS) by proteasome inhibitors has been suggested to be of importance for cytotoxicity of these agents. Interestingly, we observed induction of Hmox-1, a gene associated with oxidative stress, by all 6 agents tested (Fig. 4B). Involvement of oxidative stress therefore seems plausible with regard to the cytotoxicity of these different drugs.

As previously reported, bortezomib and b-AP15 induced strong expression of Hsp70B, a highly inducible form of Hsp70. The strong accumulation of polyubiquitinated proteins by HRF-3 and compounds such as ES-1 was expected to induce expression of this chaperone, but only limited Hsp70B induction was observed. Heat shock proteins have been described to interact directly or indirectly with the proteasome, presumably to aid proteasomal degradation. Chaperones are known to protect cells from proteotoxic stress and it is
interesting to speculate whether the lack of induction of Hsp70B contributes to the cytotoxicity of compounds such as HRF-3 and ES-1.

When investigating the cytotoxic activity of HRF-3, myeloma cell lines and leukaemia cell lines were the most sensitive, in agreement with the known cytotoxic activity of bortezomib. The cytotoxic activity of HRF-3 was also investigated in patient tumour cells since it has previously been shown that in vitro activity of primary tumour samples can predict phase II activity in patients. Compared to cell lines, the disease-specific phenotype of primary tumour cells is largely intact with respect to drug sensitivity, thus primary cells may therefore be better predictors of clinical activity. Another difference is that primary tumour samples are non-proliferating and generally more drug resistant compared to highly proliferative cell lines. Surprisingly, in our study, primary tumour samples were more sensitive to HRF-3 than the cell lines. When studying the activity of bortezomib in the same patient samples as HRF-3, a similar activity profile was found.

### Table 1 Summary of IC50 values in human tumour cell lines

| Cell line       | Origin                  | IC50 μM (95% CI) |
|-----------------|-------------------------|-----------------|
| RPMI 8226       | Myeloma                 | 1.3 (1.1–1.5)   |
| 8226/Dox40      | Myeloma                 | 2.8 (2.4–3.1)   |
| NCI-H929        | Myeloma                 | 0.61 (0.54–0.69) |
| CCRF-CEM        | T-Cell leukaemia        | 1.8 (1.6–2.0)   |
| Kasumi-1        | Acute myeloid leukaemia | 1.9 (1.7–2.3)   |
| HL-60           | Acute myeloid leukaemia | 2.5 (2.3–2.8)   |
| HCT116          | Colon carcinoma         | 5.8 (5.4–6.2)   |
| MelJuSo         | Melanoma                | 5.4 (5.1–5.8)   |

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In conclusion, we here describe a novel inhibitor of the UPS that shows cytotoxic activities on human tumour cells. The compound was found to induce cellular polyubiquitin accumulation in the absence of efficient proteasome blocking. The exact mechanisms of action of HRF-3, as well as other UPS inhibitors such as piperlongumine and physalin B, will be interesting to evaluate. The findings suggest that chemical biology will be a powerful tool in dissecting details on the UPS that are presently unclear.

Experimental procedures

Compounds

A chemical library consisting of 10 000 drug-like small molecules was purchased from Chembridge (San Diego, CA, USA). HRF-3 (Phosphoric acid, 2,3-dihydro-1,1-dioxido-3-thienyl diphenyl ester) was purchased from Chembridge Hit2lead (San Diego, CA, USA) and bortezomib was purchased from LC Laboratories (Woburn, MA, USA). The 19S inhibitor b-AP15 was synthesized by OncoTargeting AB (Uppsala, Sweden) (also available from Boston Biochem, Cambridge, MA). The VCP/P97 inhibitor NMS859 was purchased from Xcessbio. Piperlongumine was purchased from Sigma-Aldrich (Stockholm, Sweden) and ES1 was a gift from Prof. Stephen High, University of Manchester. Dimethyl sulfoxide (DMSO) was used as the solvent for stock preparations and all further dilutions were performed in sterile water or phosphate buffered saline (PBS) with a maximum of 1% DMSO in the cell culture. For experiments, 384-well microplates (Nunc) and 96-well plates (Greiner BioOne) were prepared with 5 µl of respective compound in replicate at ten times the desired final drug concentrations using a Biomek 2000 pipetting station (Beckman Coulter Inc, Fullerton, CA, USA). The plates were stored at −70 °C until further use.

Cancer cell lines

The cell lines used for cytotoxicity measurements were: the myeloma cell lines NCI-H929 and RPMI-8226, the acute myeloid leukaemia cell lines Kasumi-1 and HL-60 and the colon carcinoma cell line HCT116, all obtained from the American Type Culture collection (ATCC, Rockville, MD, USA). The 8226/Dox40 cell line, which is selected for P-gp mediated doxorubicin resistance, was a kind gift from WS Dalton, Department of Medicine, Arizona Cancer Center, University of Arizona, Tucson, AZ, USA. The T-cell leukaemia cell line CCRF-CEM was a kind gift from WT Beck, Department of Pharmacology, College of Medicine, University of Tennessee, Memphis, TN, USA. All cell lines were cultured in RPMI 1640 medium, with the exception of HCT116, which was cultured in McCoy’s 5A medium. The cell plating density was 5000 cells per well for all cell lines. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2, and cell culturing medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 µg ml−1 streptomycin and 100 U ml−1 penicillin (Sigma-Aldrich, Stockholm, Sweden). The stably transfected HEK 293 ZsGreen Proteasome Sensor cell line expressing the ZsProSensor-1 protein (BD Bioscience, San Jose, CA, USA), used for screening of proteasome inhibitors, was grown in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented as above with addition of the antibiotic G418 at 0.2 mg ml⁻¹ to select stably transfected cells. The cell line MelJuSo UbG76V-YFP (a kind gift from Professor Nico Dantuma, Karolinska Institutet), a human melanoma cell line expressing ubiquitin fused to yellow fluorescent protein (YFP), was grown in DMEM.

Measurement of cytotoxicity

Cell survival was measured using the non-clonogenic fluorometric microculture cytotoxicity assay (FMCA), described previously in detail. The FMCA is based on the measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes. For experiments, cells were seeded in drug-prepared 384-well microplates and incubated in a humidified incubator at 37 °C 5% CO2 for 3 days. Columns without drugs served as controls for normal growth and cell containing media served as blanks. After incubation, medium and drugs were aspirated, the cells were washed twice with PBS, and FDA was added. After 50–70 min of incubation, the fluorescence, which is proportional to the number of attached living cells, was measured at 485/520 nm in a fluorometer (Fluostar Optima, BMG Technologies, Germany). Cell survival is presented as the Survival Index (SI), defined as the mean fluorescence in drug treated wells as a percentage of untreated wells, with blank values being subtracted. The fluorescence in the control wells had to be at least five times the mean blank value (signal/noise ratio) and a coefficient of variation (CV) of <30% in the control wells to be defined as a positive hit. The FMCA was used for cell survival analysis throughout.

Screening for cytotoxic compounds in HCT116 colon carcinoma cells

The chemical library was screened for cytotoxic compounds using the FMCA as described above with small modifications. HCT116 colon carcinoma cells were seeded in flat-bottomed 96-well plates (Nunc) and allowed to attach, prior to drug exposure for 72 h. Compounds were screened at a concentration of 25 µM and the criterion for a hit compound was a cell survival below 35%.

Screening for UPS inhibitors

The screening for UPS inhibitors was performed as in ref. 14 with minor changes, using the HEK 293 ZsGreen Proteasome Sensor Cell Line, engineered to express an ornithine decarboxylase (ODC)-fusion green-fluorescent protein that is degraded by the proteasome without the need for prior ubiquitination. The cells were plated in black optically clear bottom ViewPlates (Perkin-Elmer, Waltham, MA, USA), left to attach and then treated with 5 µM of compounds for 16 h. Then the cell nuclei were stained with Hoechst 33342 and the plates were analysed in an ArrayScan II instrument (Thermo Fisher Scientific, Waltham, MA, USA). The criterion for a hit compound was
a generated fluorescence of three standard deviations (SD) above the background fluorescence.

Connectivity map analysis
Data from Connectivity Map (www.broadinstitute.org/cmap/) were downloaded and visualized using TMV4. The 30 most up-and down-regulated genes after HRF-3 treatment were selected from the two instances of HRF-3-treated MCF-7 cells (the ChemBridge ID for HRF-3 is 5155877). The expression values for the selected genes after treatment with the protease inhibitors MG-262, 15β prostaglandin J2 (15β-PGJ2), thiorstrepton and withaferin A were retrieved and visualized along with HRF-3 in a heat map format.

Live-cell imaging of UPS-activity
MelJuSo UbG76V-YFP cells were plated in black optically clear bottom ViewPlates (Perkin-Elmer) overnight and then treated with HRF-3 at 5 μM. Bortezomib at 10 μM was used as a positive control. Treatment with UPS-inhibiting compounds leads to accumulation of YFP in the cells, and the generated fluorescence from the ubiquitin construct was continuously detected and studied in an IncuCyte FLR (Essen BioScience Inc., Ann Arbor, MI, USA).

Activity assay for 20S proteasome
Quantification of the chymotrypsin activity of the 20S proteasome in the presence of HRF-3 was determined using a cell free 20S proteasome assay kit (BostonBiochem, Cambridge, MA, USA) according to the manufacturer’s instructions. Briefly, 20S activity was measured by adding HRF-3 or the known 20S inhibitor bortezomib to 20S erythrocyte proteasomes in reaction and activation buffer (25 mM HEPES, 0.5 mM EDTA, pH 7.6 and 0.03% SDS). The compounds were incubated with 20S enzyme solution for 15 min at 37 °C before the substrate solution (10 μM Suc-LLVY-AMC) was added. The increase in fluorescence was measured every 3rd minute using excitation and emission wavelengths of 340 and 450 nm, respectively. Monitoring the increase in fluorescence over time allowed quantification of the enzymatic activity.

Western blot analysis
Protein extraction from cells was performed with RIPA lysis buffer (50 mM Tris–HCL pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton-X, 0.1% SDS) freshly supplemented with protease inhibitor cocktail (Sigma-Aldrich), 10 mM N-ethylmaleimide (NEM; EMD Chemicals). Protein samples were collected by centrifugation at 13 000g for 15 min at 4 °C and the concentration was measured by the Bradford (Bio-Rad, Richmond, CA, USA) assay. Proteins (10 μg) were denatured under reducing conditions (1% β-mercaptoethanol) in NuPAGE sample buffer (Invitrogen, Carlsbad, CA) and subjected to gel electrophoresis, followed by transfer to polyvinylidene difluoride (PVDF; Hybond-P, Amersham, UK) membranes. Membranes were blocked for 1 h in 5% non-fat dry milk (Bio-Rad) in Tris Buffer saline Tween-20 (TBS-T) at room temperature and probed with primary antibodies overnight at 4 °C and with horseradish peroxidase-labelled secondary antibodies (anti-mouse IgG and anti-rabbit IgG) for 1 h at room temperature. For HRP detection, an ECL chemiluminescence kit (Amersham) was used according to the manufacturer’s instructions and membranes exposed to the X-ray film. Anti-UbK48 (Apu2; Millipore, Temecula, California), anti-HspA6 (Abcam), anti-heme oxygenase 1 (BD Transduction Laboratories), anti-p21 (H-164, Santa Cruz Biotechnology), anti-GFP (4B10; Cell Signalling) and anti-β-actin (Sigma-Aldrich) primary antibodies were used.

Patient tumour cells
Tumour samples were obtained by routine surgery, diagnostic biopsy or bone marrow/peripheral blood sampling approved by the ethical committee at Uppsala University (Dnr 2007/237). The samples were obtained from patients with colon carcinoma (n = 5), pseudomyxoma peritonei (PMP) (n = 7), acute myeloid leukaemia (AML) (n = 7) and chronic lymphatic leukaemia (CLL) (n = 9). PMP is a mucinous tumour within the peritoneal cavity where the primary tumour predominately is a neoplasm of the appendix, or less commonly from an ovarian tumour. Tissue from solid tumour samples was processed by mincing with scissors, and tumour cells were isolated by collagenase dispersion followed by purification using Percoll density gradient centrifugation (GE Healthcare, Uppsala, Sweden). Leukemic cells were isolated from bone marrow or peripheral blood using density gradient centrifugation in 1.007 g ml−1 Ficoll-Paque (GE Healthcare). Cell viability was determined by Trypan blue exclusion, and the proportion of tumour cells (>70%) was assessed by inspection of May-Grünwald-Giemsa-stained cyto spun preparations. The medium used throughout was RPMI 1640 supplemented as mentioned above and the cell plating density was 5000, 20 000 and 40 000 cells per well for solid tumour samples, AML and CLL, respectively.

Statistics
Statistical calculations were performed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Dose–response data were processed using log-linear interpolation to obtain log IC50 values (the concentration resulting in 50% survival compared with control). IC50 values are presented as the mean IC50 in the repeated experiments, with 95% confidence intervals. In cases where an IC50 could not be determined, the IC50 value was reported as the highest or lowest concentration tested. For patient samples, the median IC50 values are used throughout.

Acknowledgements
The skilful technical assistance of Kristin Blom, Anna-Karin Lannerård, Lena Lenhammar and Annika Jonasson is gratefully acknowledged. We thank Stephen High, University of Manchester, for the gift of ES-1. This work was supported by Cancerfonden, Radiumhemmets forskningsfonder, Veten skapsrådet and Strategiska Forskningsstiftelsen.
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