Identification of proteasome inhibitors using analysis of gene expression profiles

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ABSTRACT

Inhibitors of the 20S proteasome such as bortezomib (Velcade®) and carfilzomib (Kypriolis®) are in clinical use for the treatment of patients with multiple myeloma and mantle cell lymphoma. In an attempt to identify novel inhibitors of the ubiquitin-proteasome system (UPS) used the connectivity map (CMap) resource, based on alterations of gene expression profiles by perturbagens, and performed COMPARE analyses of drug sensitivity patterns in the NCI60 panel. Cmap analysis identified a large number of small molecules with strong connectivity to proteasome inhibition, including both well characterized inhibitors of the 20S proteasome and molecules previously not described to inhibit the UPS. A number of these compounds have been reported to be cytotoxic to tumor cells and were tested for their ability to decrease processing of proteasome substrates. The antibiotic thioestrent and the natural products celery, curcumin induced strong accumulation of polyubiquitinated proteasome substrates in exposed cells. Other compounds elicited modest increases of proteasome substrates, including the protein phosphatase inhibitor BCI–Cl and the farnesyltransferase inhibitor manumycin A, suggesting that these compounds inhibit proteasome function. Induction of chaperone expression in the absence of proteasome inhibition was observed by a number of compounds, suggesting other effects on the UPS. We conclude that the combination of bioinformatic analyses and cellular assays resulted in the identification of compounds with potential to inhibit the UPS.

1. Introduction

Cancer is a disease characterized by the occurrence of a large number of genetic and epigenetic alterations (da Silva-Diz et al., 2018). The clinical efficacy of drugs that inhibit mutant enzymes and receptors is hampered by the development of clones expressing variant target proteins, resulting in treatment failure (van der Wekken et al., 2016; Wagle et al., 2011). Compounds with mechanisms relying on polypharmacology for their antineoplastic activity may therefore be beneficial (Anighoro et al., 2014). Natural products display considerable structural diversity (Chen et al., 2017; Stratton et al., 2015), facilitating interactions with a variety of targets (Mishra and Tiwari, 2011). Natural products do, however, frequently contain Pan Assay INterference compounds (PAINS) motifs (Baell, 2016), expected to convey wide-spread activity in biochemical screens (Baell, 2016; Rodrigues et al., 2016). Approximately one sixth of all natural products contain Michael acceptors (Rodrigues et al., 2016), structures that are often required for their biological activity (Adams et al., 2012; Oliveira et al., 2015; Zhang et al., 2004). Many natural products with Michael acceptor functionalities show antineoplastic activity in animal models and do not seem to be generally toxic. Whether such compounds may serve as leads for drug design is controversial since the Michael acceptor function will be expected to lead to side effects.

The proteasome is the major protein degradation system in eukaryotic cells (Goldberg, 2007; Hershko and Ciechanover, 1998). Proteasomal degradation is necessary for removal of potentially toxic misfolded proteins and also serves nutritional roles under starvation conditions by recycling amino acids (Elharar et al., 2014; Suraweera et al., 2012). Tumor cells, and in particular myeloma cells, display increased sensitivity to proteasome inhibition, likely due to elevated levels of defective ribosomal products (Meister et al., 2007; Obeng et al., 2006). The proteasome contains a 20S core particle harboring the enzymatic cleavage activity and one or two regulatory particles. Proteins are tagged by ubiquitin for subsequent degradation by the proteasome. Protein ubiquitination is a reversible process where deubiquitinases (DUBs; ubiquitin isopeptidases) are able to remove ubiquitin from substrates by cleaving...
the isopeptide bond between the C-terminus of ubiquitin and a lysine residue on a target protein (Hershko and Ciechanover, 1998; Singh and Singh, 2016). The majority of DUBs contain functional cysteines (Komander et al., 2009) and expected to be druggable. In humans, three DUBs are associated with the 19S regulatory particle of the proteasome, two of which (UCHL5/Uch37 and USP14/Ubp6) are cysteine proteases.

Analysis of gene expression profiles is a powerful strategy to generate hypotheses with regards to the potential mechanisms of action of small molecules (Lamb et al., 2006; Subramanian et al., 2017). The Broad Institute LINCS Center for Transcriptomics has generated more than one million transcriptomic profiles that can be used for this purpose (Subramanian et al., 2017). Proteasome inhibitors are of large clinical interest and used for clinical management of multiple myeloma (Chim et al., 2018) and we here examined whether novel proteasome inhibitors can be identified by examination of gene expression profiles.

2. Materials and Methods

2.1. CMap analysis

The Broad Institute CMap database was accessed through https://clue.io/touchstone. Perturbagen types (compound or gene knockdown) were selected following by “View connections”. This procedure results in lists of compounds ranked according to the degree of similarities of gene expression patterns that are induced after drug exposure according to taur-scores. These scores correspond to the fraction of reference gene sets with a greater similarity to the perturbagen than the query (https://clue.io/connectopedia/connectivity_scores). Taur-scores of +90 or higher are considered as strong scores. We either used proteasome pathway loss-of-function (knock-down of PSMA1, PSMA3, PSMA5, PSMA7, PSMB1, PSMB2, PSMB5, PSMB7 and PSMD8) proteasome catalytic subunits (PSB1, PSB2 and PSMB5) (Fig. 1 and Suppl. Fig. 1). The proteasome inhibitor profile includes compounds z-Leu3-VS, MG-132 and MLN2238.

2.2. Generation of L1000 firework plots

L1000 Firework plots (Wang et al., 2018) were generated using the L1000FWD tool (http://amp.pharm.mssm.edu/L1000FWD/). Data was limited to gene expression from experiments using MCF7 cells, and clustering using tSNE. Each node represents a drug-induced gene expression signature (Wang et al., 2018). Clustering of the compounds derived from the CMap analysis were accessed through the tool’s database. Clustering of compounds from our prior screen19 were submitted by entering the ranked list of 50 most up- and downregulated genes relative to control for the respective compound. For clarity, yellow circles indicating an experiment point in the cluster are circled in black.

2.3. Classification of compounds

Physicochemical descriptors were collected using the SwissAdme website (http://www.swissadme.ch/index.php). Promiscuous hitters in bioassays were scored using the http://postilla.health.unm.edu/tomcat t/badapple/badapple website by entering canonical SMILES from PubChem. The sensitivity of cells in the NCI60 panel to various compounds was analyzed using data from NCI (https://dtc.cancer.gov/dtpstandar d/dwindex/index.jsp). GI50 values were compared pairwise and Spearman correlation coefficients were calculated. Hierarchical clustering was performed using the CMap database accessed through clue.io. Genetic signatures were uploaded to the tool by providing a ranked list of 150 most up- and downregulated genes relative to control for the respective compound in the “query” tool (clue.io). Hierarchical clustering was performed using 1-pearson score method.

2.4. Cell culture and image analysis

The proteasome reporter cell line MelJuSo Ub767V-YFP (Menendez-Benito et al., 2005) and HCT116 cells were cultured in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum. Cells were maintained at 37 °C in 5% CO2. For evaluation of antiproliferative effects cells were seeded in 96 well-plates and compounds were added after 24 h. After an additional 48 h, 3-(4,5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added at a final concentration of 0.05 mg/ml per well for 3 h and absorbance measured at 550 nm. All compounds tested in this work were dissolved in dime- thylsulphoxide. A maximum final concentration of 0.5% DMSO was reached in cell cultures; control wells received solvent only. The following concentrations were used: b-AP15: IC50 0.49 μM, IC90 0.57 μM; bortezomib: IC50 0.20 μM, IC90 0.32 μM; BCI-Cl: IC50 3.42 μM, IC90 5.49 μM; z-Leu3-VS: IC50 2.11 μM, IC90 2.39 μM; auranofin: IC50 1.67 μM, IC90 3.56 μM; withaferin A: IC50 2.11 μM, IC90 2.37 μM; thio- strepton: IC50 0.25 μM, IC90 0.33 μM; parthenolide: IC50 16.33 μM; piperlongumine: IC50 4.26 μM; BNTX maleate: IC50 1.95 μM; tegaserod: IC50 6.43 μM; 17-DMAG: IC50 9.57 μM; MG-132: IC0.16 μM; NSC632839: IC1 1.89 μM; dacomastat: IC50 12.7 μM; SA-792709: IC50 22.4 μM; SA-1478: IC50 9.55 μM; MLN4924: IC50 0.59 μM; JTC-801: IC50 3.34 μM; curcumin: IC50 29 μM; celastrol: IC0.141 μM; manumycin A: IC50 24.7 μM; curcubatcin: IC50 3.29 μM; radicicol: IC50 7.3 μM; CA-074-Me: IC50 36.4 μM.

Analysis of cellular functional proteasome activity was performed using MelJuSo Ub767V-YFP cells transfected with a Nuclear Red marker (Essen BioScience Inc., Ann Arbor, MI). The Ub767V-YFP reporter is normally degraded by the proteasome and will accumulate in cells when proteasome function is blocked (Menendez-Benito et al., 2005). Cells were seeded in 96-well plates and allowed to attach overnight prior to treatment. Compounds were used at IC50 concentrations or at a concentration range of 0.1 μM, 1 μM and 10 μM. Immediately upon addition of compound, cells were placed in an Incucyte® ZOOM instrument (Essen BioScience) and images were collected every hour. Red nuclei were tracked for cell counting, and fluorescent signals indicate accumulation of the Ub767V-YFP fusion protein.

2.5. Western blot analysis

MelJuSo Ub767V-YFP cells were treated with the indicated concentrations of compounds for 6 h. Cells were lysed using RIPA buffer (Cell Signaling Technology, Danvers, Mass) supplemented with protease and phosphatase inhibitor (Invitrogen, Carlsbad, Calif). Lysates were diluted to 1 μg/μl protein in reducing NuPAGE™ SDS Sample Buffer (Invitrogen). 15 μg of protein per sample were resolved by SDS-PAGE using Tris-Acetate 3–8% gels for the analysis of lysine-48-linked polyubiquitin (K48 poly-Ub) proteins and Hsp70B’. Proteins were transferred onto nitrocellulose membranes which were incubated overnight with primary antibodies, washed and incubated with HRP-conjugated anti-rabbit IgG (7074S) or anti-mouse IgG (7076p2) (Cell Signaling Technology, Danvers, Mass) for 1h. Antibodies used were: K48-linked polyubiquitin (05–1307) (Apu2, Millipore), Hsp70B’ (HPA028549, Sigma-Aldrich), β-actin (AS516, Sigma-Aldrich; 1:10,000).

2.6. 20S proteasome catalytic activity

For analysis of 20S catalytic activity, MelJuSo Ub767V-YFP cells were treated with indicated concentrations of compounds for 6 h. Full protein extracts were diluted to 1 μg/μl in reaction buffer (25 mM Heps, 0.5 mM EDTA, 0.03% SDS) and then incubated with Suc-LYV-2R110 substrate. Fluorescent signals formed by cleavage of the substrate were measured over time using a Tecan Infinite 200 reader equipped with 498 nm excitation and 520 nm emission filters.
2.6.1. Proteasome purification

Proteasomes were affinity purified from cells exposed to different compounds using ProteaSelect-HEK293_Bio-Rpn11 cells (from Ubiquigent, Scotland). These cells express a 6-His/Biotin-tagged PSMD14/Rpn11 proteasome subunit that can be retrieved by high-affinity streptavidin binding followed by TEV cleavage (Wang et al., 2007).

3. Results

3.1. Identification of potential proteasome inhibitors using connectivity analysis

The association between alterations in gene expression patterns induced by gene deletion or by exposure to small molecules is referred to as "connectivity" (Lamb, 2007). We used the Broad Institute Connectivity Map (CMap) resource to search for compounds that induce phenotypic responses, i.e. showing connectivity, similar to knock-down of transcripts encoding proteasome subunits or pharmacological proteasome inhibition (Fig. 1). For further information on the selection of knock-downs, see Supplementary Figs. 1 and 2. The 50 highest ranking compounds of a total of 2429 are listed in Fig. 1. All these compounds had tau-scores higher than +90 and were considered candidate inhibitors of the UPS (see Fig. 1 legend). Compounds with documented proteasome inhibitory activity such as z-leu3-VS, MLN-2238 and MG-132 were top-ranked on both lists. The α,β-unsaturated compound NSC632839 also showed strong connectivity to proteasome inhibition. NSC632839 is a structural analogue of b-AP15 (D’Arcy et al., 2011), RA-9 (Coughlin et al., 2014) and EP24 (He et al., 2018) and has been shown to inhibit DUB activity (Aleo et al., 2006). These compounds have been demonstrated to induce gene expression profiles that are similar to that induce by bortezomib, a major difference being the stronger induction of chaperone genes by b-AP15 and related compounds (D’Arcy et al., 2011; Tomassela et al., 2016). The structures of the molecules discussed here are shown in Fig. 2 (for SMILES see Suppl. Table 1). A number of compounds that have not been reported to display proteasome inhibitory activity were also highly ranked, including BCI-Cl (an inhibitor of dual-specificity phosphatase 6 (Dusp6) (Molina et al., 2009)), cucurbitacin-I/JSI-124 (a JAK/STAT3 inhibitor (Blaskovich et al., 2003)), the opioid receptor antagonists BNTX (7-benzylidene-naltrexone) and JTC-801 (Portoghese et al., 1992; Yamada et al., 2002). Compounds previously described to inhibit the UPS such as auranofin (Liu et al., 2014), disulfiram (Lovborg et al., 2006), celestrol (Yang et al., 2006) and curcumin (Zhou et al., 2013) ranked lower than 50 on the lists of connectivity. We finally note that 16 of the 50 highest ranking compounds listed in Fig. 1 contain α,β-unsaturated carbonyls (flagged by yellow boxes) functioning as Michael acceptors. The CMap analysis raises the possibility that a number of commonly used small molecule inhibitors may affect the UPS, which would be problematic and confound the results of studies using these compounds as biological probes.

We selected 29 molecules for further studies (Table 1, Suppl. Table 1). Some of these compounds (auranofin, curcumin, celestrol and disulfiram) were included based on previous studies showing UPS inhibition (Banerjee et al., 2018; Liu et al., 2014; Lovborg et al., 2014; Zhang et al., 2019).
3.2. Examination of gene expression signatures

Connectivity was further analyzed using L1000 firework displays (Wang et al., 2018) (clue.io) (see Suppl. Fig. 3). To facilitate comparisons with other compounds (see below) the analysis was restricted to MCF-7 cells. DNA damaging compounds induced related signatures, and the microtubule inhibitors paclitaxel and vinblastine also induced partially overlapping signatures (Fig. 3). The signatures of the proteasome inhibitors bortezomib and MG-132 were quite distinct from those of these reference drugs (Fig. 3). Furthermore, the DUB inhibitors b-AP15 and NSC-632839 displayed signatures similar to proteasome inhibitors. Most signatures of compounds identified by CMap contained one or a few nodes related to bortezomib and MG-132, but the patterns were often more diverse. Two compounds, the HDAC inhibitor dacinostat and the metalloprotease inhibitor SA-1478088, did not display any node in the bortezomib/MG-132 region.

### Table 1

<table>
<thead>
<tr>
<th>Compound/drug</th>
<th>LogP</th>
<th>PAINS</th>
<th>Badapple</th>
<th>Michael acceptor</th>
<th>Gene expression induction#</th>
</tr>
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<tr>
<td>AA-COCF3</td>
<td>6.93</td>
<td>No</td>
<td>U</td>
<td>No</td>
<td>+++</td>
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<tr>
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<td>No</td>
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<td>Yes</td>
<td>++</td>
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<tr>
<td>BCI-Cl</td>
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<td>++</td>
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<tr>
<td>BNTX</td>
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<td>++</td>
</tr>
<tr>
<td>Bortezomib</td>
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<td>No</td>
<td>Moderate</td>
<td>No</td>
<td>++</td>
</tr>
<tr>
<td>CA-074-Me</td>
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<td>No</td>
<td>Moderate</td>
<td>No</td>
<td>++</td>
</tr>
<tr>
<td>Celastrol</td>
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<td>Yes</td>
<td>U</td>
<td>Yes</td>
<td>+</td>
</tr>
<tr>
<td>Curcumin</td>
<td>2.96</td>
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<td>Moderate</td>
<td>Yes</td>
<td>++</td>
</tr>
<tr>
<td>Curcubitacin I</td>
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<td>Yes</td>
<td>U</td>
<td>Yes</td>
<td>++</td>
</tr>
<tr>
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<td>High</td>
<td>No</td>
<td>–</td>
</tr>
<tr>
<td>Devacepside</td>
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<td>No</td>
<td>High</td>
<td>No</td>
<td>+</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>3.25</td>
<td>No</td>
<td>U</td>
<td>No</td>
<td>+</td>
</tr>
<tr>
<td>17-DMAG</td>
<td>5.15</td>
<td>Yes</td>
<td>Low</td>
<td>Yes</td>
<td>+</td>
</tr>
<tr>
<td>Flavokawain-B</td>
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<td>No</td>
<td>High</td>
<td>Yes</td>
<td>+</td>
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<td>ITC-801</td>
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<td>No</td>
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<td>No</td>
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</tr>
<tr>
<td>JLK-6</td>
<td>1.95</td>
<td>No</td>
<td>Low</td>
<td>No</td>
<td>–</td>
</tr>
<tr>
<td>Manumycin A</td>
<td>3.18</td>
<td>No</td>
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<td>MG-132</td>
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<tr>
<td>MLN4924</td>
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<td>+</td>
</tr>
<tr>
<td>NSC-632839</td>
<td>5.15</td>
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<td>High</td>
<td>Yes</td>
<td>++</td>
</tr>
<tr>
<td>PGJ2</td>
<td>4.44</td>
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<td>U</td>
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<td>++</td>
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<tr>
<td>Piperlongumine</td>
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<td>Low</td>
<td>Yes</td>
<td>++</td>
</tr>
<tr>
<td>Radicicol</td>
<td>2.43</td>
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<td>Low</td>
<td>No</td>
<td>++</td>
</tr>
<tr>
<td>SA-792709</td>
<td>5.37</td>
<td>No</td>
<td>High</td>
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<td>+</td>
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<tr>
<td>SA-1478088</td>
<td>3.03</td>
<td>No</td>
<td>High</td>
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<td>+</td>
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<tr>
<td>Tegaserod</td>
<td>2.42</td>
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<td>High</td>
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<td>+</td>
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<tr>
<td>Thiostrepton</td>
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<td>High</td>
<td>No</td>
<td>+</td>
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<td>Withaferin A</td>
<td>4.97</td>
<td>No</td>
<td>Low</td>
<td>Yes</td>
<td>+</td>
</tr>
</tbody>
</table>

LogP = consensus LogP from SwissAdme. PAINS were scored using SwissAdme and promiscuous hitters in bioassays using the Badapple tool (Yang et al., 2016). Some scaffolds are not represented in Badapple (U: unknown). # Gene expression profiles were retrieved as described in Fig. 4. Scoring was as follows: chaperones: ++: 5 genes induced; +++: 3–4 genes induced; +: 1–2 genes induced; immediate early genes (IE): ++: 4 genes induced; +/-: 1–3 genes induced; ER stress: ++: ≥2 genes induced; +: one gene induced. SA-1478088 is also known as ARP-101. AA-COCF3: arachidonyl-trifluoro-methane. 17-DMAG (alvespimycin, a geldanamycin analogue). Canonical SMILES for these compounds are shown in Suppl. Table 1.
Analyses of gene expression in different cell lines exposed to these compounds is available from L1000FWD (see Material and Methods). HMOX1 (heme oxygenase 1, an Nrf-2 target and a marker of oxidative stress (Hamamura et al., 2007)), chaperones, immediate early response genes (JUN and FOS family) and ER stress markers were induced by bortezomib and MG-132 (Fig. 4, different shades of red). Other genes were down-regulated (Fig. 4, different shades of blue), such as cyclin genes, likely as a consequence of inhibition of cell proliferation. These results are consistent with previous data (Fribley et al., 2004). We focused on upregulated genes and the profiles for some compounds are shown in Fig. 4 and the results summarized in Table 1. With the exception of dacinostat and the serotonin agonist tegaserod, all compounds induced chaperone expression in most cell lines tested. HMOX1 was also widely induced, dacinostat being the only compound not reported to induce the expression of this gene. The natural products curcumin, manumycin A, parthenolide, piperlongumine, and withaferin A all induced strong chaperone expression, HMOX1, immediate early response genes and ER stress markers. The Dusp6 inhibitor BCI–Cl was reported to induce the expression of five different Hsp70 chaperones, BAG3 and HSP40. Furthermore, in addition to HMOX1 and immediate early genes, BCI–Cl also induced the ER-stress markers HERPUD1 and DDIT3 (CHOP). Finally, we noticed that the Hsp90 inhibitors 17-DMAG and radicicol induced the expression of HMOX1 and chaperones, but differed with regard to induction of immediate early genes and ER stress markers. We conclude that the gene expression analyses resulted in the identification of a number of potential inhibitors of the UPS.

3.3. COMPARE analysis

A number of the compounds listed in Table 1 have been demonstrated to display in vivo anticancer activity using animal models. Information with regard to sensitivity of tumor cells in the NCI60 panel of tumor cell lines is available for some of these compounds (dtp.nci.gov). One criterium for specificity of mechanism of action is that the...
sensitivities of cell lines in the panel will differ (i.e. generally cytotoxic agents often show similar IC$_{50}$ values over the panel) (Boyd and Paull, 1995; Wallqvist et al., 2003). The Michael acceptor-containing compounds induce diverse patterns of proliferation inhibition over the NCI$_{60}$ cell panel (in average a span of 1.84 ± 0.35 (log$_{10}$) between the most and least sensitive cell line) (Table 2). This difference in NCI$_{60}$ inhibition is in fact similar or even larger compared to that of a number of natural products not containing Michael acceptors that are anticancer drug candidates (Lin et al., 2020) (span of difference in GI$_{50}$ in the NCI$_{60}$ cell line panel (log$_{10}$): 1.42 ± 0.54).

The GI$_{50}$ values for different compounds in the NCI$_{60}$ cell line panel can be pairwise compared and expressed as correlation coefficients (COMPARE analysis). A high correlation between the sensitivities of two compounds over the panel may reflect similar mechanisms of action, although factors such as drug uptake and metabolism will affect drug sensitivities and affect the results. Four compounds showed significant correlations to bortezomib and three showed significant correlations to b-AP15 (Table 2). BCI–Cl and withaferin A showed significant correlations to both bortezomib and b-AP15 (Table 2). The ranking of compounds in the COMPARE analysis and the CMap analysis for proteasome inhibitor connectivity showed no correlation ($r = -0.14$; Table 1 legend).

3.4. Analysis of inhibition of proteasome function

A central question is whether any of the compounds and drugs identified here inhibit proteasome function. The PAINS characteristics of some of the compounds prompted us not to primarily depend on in vitro assays to address this question but to examine accumulation of K48-linked polyubiquitinated proteins in exposed cells. Proteasome function is well known to be essential for the viability of tumor cells (Nijhawan et al., 2012; Richardson et al., 2005) and compounds that were not

![Fig. 4. Gene expression profiles. Shown are expression data induced by different compounds in tumor cell lines using Clustergrammer heatmaps (Fernandez et al., 2017). Different cancer cell lines are represented in columns (MCF-7 high-lighted) and genes as rows. Genes that are upregulated are shown in different shades of red and downregulated genes in blue. Chaperones are boxed in black, HMOX-1 in blue (dashed), immediate early genes in green and genes induced by ER stress in yellow. Data are from L1000FWD (amp.pharm.mssn.edu/dmoa/search_drug).](image-url)
Table 2

Sensitivities of cell lines in the NCI60 cell line panel to Michael acceptor compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ΔNCI60 inhibition (log₁₀)</th>
<th>Correlation coefficient</th>
<th>p-value</th>
<th>Compound</th>
<th>ΔNCI60 inhibition (log₁₀)</th>
<th>Correlation coefficient</th>
<th>p-value</th>
</tr>
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<tr>
<td>Parthenolide</td>
<td>1.3</td>
<td>0.483</td>
<td>0.0008</td>
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<td>0.374</td>
<td>0.0049</td>
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<td>BCI-Cl</td>
<td>2.2</td>
<td>0.382</td>
<td>0.0003</td>
<td>Withaferin A</td>
<td>2.1</td>
<td>0.364</td>
<td>0.0063</td>
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<tr>
<td>Celastrol</td>
<td>1.7</td>
<td>0.349</td>
<td>0.0078</td>
<td>15Δ-PGJ2</td>
<td>N.A.</td>
<td>0.322</td>
<td>0.018</td>
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<tr>
<td>Withaferin A</td>
<td>2.1</td>
<td>0.261</td>
<td>0.050</td>
<td>Celastrol</td>
<td>1.7</td>
<td>0.224</td>
<td>0.100</td>
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<td>Cucurbitacin i</td>
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<td>0.208</td>
<td>0.127</td>
<td>Parthenolide</td>
<td>1.3</td>
<td>0.166</td>
<td>0.282</td>
</tr>
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<td>b-AP15</td>
<td>3.7/1.9*</td>
<td>0.187</td>
<td>0.172</td>
<td>Cucurbitacin i</td>
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<td>0.131</td>
<td>0.350</td>
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<td>0.322</td>
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</tr>
</tbody>
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GI₅₀ values for the NCI60 panel were obtained from the dtp.nci.gov website. The term ΔNCI60 inhibition was calculated as the 10-log difference between proliferation inhibition between cell lines in the panel (i.e. CCRF cell line being most sensitive to BCI-Cl with a Log GI₅₀ of −6.9 M and A498 cells being the least sensitive at −4.7 M leading to a ΔNCI60 of 2.2). Correlation analysis of sensitivities to different Michael acceptor compounds and bortezomib (left) or b-AP15 (right) were calculated (correlation coefficients and p-values are shown). The ranking of compounds according to correlation coefficients with respect to bortezomib did not correlate to the ranking of these compounds in the CMap analysis (see Fig. 1, right column). * The value 3.7 is obtained when the data reported for HL60 is used (−4.0) is included. This value is likely to be erroneous and 1.9 likely to be correct.
cytotoxic at <25 μM concentrations were generally excluded. The remaining compounds were used at IC₅₀ concentrations (Fig. 5A–C). Inhibitors of the 20S proteasome (z-Leu3-VS, MG-132 and bortezomib) induced strong increases in K48-linked polyubiquitinated proteins in HCT116 colon cancer cells (Fig. 5A–C). Strong increases in polyubiquitin levels were also observed with b-AP15, thiorreton, curcumin and celastrol (Fig. 5A and B), consistent with previous reports (Banerjee et al., 2018; D’Arcy et al., 2011; Hoch et al., 2019; Sandu et al., 2015; Yang et al., 2006; Zhou et al., 2013). Moderate increases in polyubiquitinated proteins were observed in cells exposed to BCI–Cl, auranofin, withaferin A, CA-074-Me and manumycin A (Fig. 5A and B). The remaining tested compounds did not induce accumulation of polyubiquitinated proteins in HCT116 cells (Fig. 5B and C).

Almost all compounds tested were found to induce increases in the expression of the Hsp70B' chaperone (Fig. 5A, B, C). Radicicol and 17-DMAG (NSC707545, 17-demethyloxy-cytohatoxins) elicited strong increases in Hsp70B' in the apparent absence of increases in proteasome substrates. These molecules are inhibitors of Hsp90 (Mellatyar et al., 2018; Sharma et al., 1998), and have previously been shown to increase Hsp70 expression (Kuballa et al., 2015; Madrigal-Matute et al., 2010).

We examined whether selected compounds would inhibit processing of a proteasome-degraded reporter protein (UbG76V-YFP) in human melanoma cells. As expected, z-Leu3-VS and thiorreton induced rapid increases in UbG76V-YFP signals in cells (Fig. 5D). Withaferin A and BCI–Cl induced modest increases of YFP-positive cells at a concentration of 1 μM. Auranofin induced increases in the number of YFP-positive cells at a concentration of 10 μM (Fig. 5D), ~10-fold the IC₅₀ (Zhang et al., 2019). The kinetics of auranofin-induced increases differed from those of other compounds, peaking at 30 h. We also examined inhibition of 20S proteasome enzymatic activity, using a fluorescent substrate (Fig. 5E). Manumycin A showed a similar degree of inhibitory activity as z-Leu3- VS, CA-074-Me and withaferin A also showed inhibitory activity. This type of in vitro biochemical assay is, however, problematic considering the potential reactivity and PAINS characteristics of various compounds.

We wished to further document the proteasome inhibitory activity of BCI–Cl ((E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one). For this purpose we used a cell line (ProteaSelect-HEK293 Bio-Rpn11) constructed to facilitate affinity purification of proteasome (Wang et al., 2007). Proteasomes purified from cells exposed to BCI–Cl were associated with K48-linked polyubiquitinated chains (Fig. 6), consistent with accumulation of polyubiquitinated proteins at proteasomes.

3.5. Distinguishing responses of proteasome inhibitors using gene expression profiling

The mechanisms of action of the different compounds studied here clearly differ, proteasome processing only being affected by some of the compounds. The different profiles in the L1000 Firework displays may reflect such differences. To extend the analyses we examined a number of Michael acceptor compounds previously shown to inhibit processing of polyubiquitin chains at the level of the proteasome (Selvaraju et al., 2019). These compounds ("CB360", "CB383" etc.) displayed similar signatures on L1000 Firework plots with minor variations, signatures similar to those of bortezomib and b-AP15 (Fig. 3). A considerable amount of biological data is available for these compounds, including zebrafish developmental toxicity and analyses of the association between proteasome inhibition and cell death (tracing individual cells expressing the UbG76V-YFP reporter, see Fig. 7 legend) (Selvaraju et al., 2019). Using hierarchical clustering analysis, we found that two compounds, CB316 and CB688, clustered together with the DUB inhibitor b-AP15. Interestingly, cell death induced by both of these compounds was closely associated with proteasome inhibition (Fig. 7), as previously described for b-AP15 (Brnjic et al., 2014). Furthermore, similar to b-AP15, CB916 and CB688 did not induce developmental toxicity in
zebrafish at concentrations up to 20 μM (Fig. 7). However, the hierarchical clustering analysis did in general not predict the degree of association between cell death and proteasome inhibition or developmental toxicity (Fig. 7).

3.6. Compounds scored as PAINS are not necessarily generally toxic

We examined whether the Michael acceptor compounds studied here were common “hitters” in in vitro assays (PAINS; Pan Assay Interference compounds) (Baell and Holloway, 2010) or promiscuous hitters in bioassays (Badapples; bioassay-data associative promiscuity pattern learning engine) (Yang et al., 2016). Only four of the 16 Michael acceptor compounds listed in Table 1 (celastrol, cucurbitacin I, NSC-632839 and 17-DMAG) were flagged as PAINS and five compounds contained scaffolds that received high scores using the Badapple resource (Table 1). In the set of compounds identified by screening of Michael acceptors for proteasome inhibition (Selvaraju et al., 2019), 4/10 scored as PAINS and 8/10 as Badapples (Table 3). Surprisingly, of the five compounds that scored as both PAINS and Badapples, four did not exhibit developmental toxicity and all five showed a modest/strong association between proteasome inhibition and cell death (Fig. 7).

4. Discussion

Proteasome inhibitors are of large clinical interest and are used for clinical management of multiple myeloma (Chim et al., 2018). The a priori hypothesis stimulating this study was that novel proteasome inhibitors can be identified by examination of gene expression profiles elicited by small molecules. Ideally, some compounds would already be approved for clinical use and could be repurposed for cancer therapy. Using the Broad Institute LINCS database we found connectivity between proteasome inhibition (either pharmacological inhibition or knock-down of proteasome subunits) and a number of small molecules. The repurposing potential of the identified compounds appeared to be limited since almost all compounds were experimental with the possible exception of tegaserod (Zelnorm, Zelmac; a 5-HT4 agonist approved for management of irritable bowel syndrome during 2002–2007). Furthermore, despite showing strong connectivity to proteasome inhibition, most candidate compounds did not induce strong accumulation of polyubiquitinated proteins in exposed cells, comparable of that of 20S proteasome inhibitors and the DUB inhibitor b-AP15. Exceptions were the antibiotic thiostrepton and the natural products curcumin and celastrol. Thiostrepton was previously reported to inhibit proteasome activity by direct or indirect mechanisms (Banerjee et al., 2018; Hasima and Aggarwal, 2014; Yang et al., 2006; Zhou et al., 2013). Curcumin and celastrol have been shown to inhibit proteasome activity by direct or indirect mechanisms (Banerjee et al., 2018; Hasima and Aggarwal, 2014; Yang et al., 2006; Zhou et al., 2013). Curcumin inhibits the chymotrypsin activity of the 20S proteasome (Gupta et al., 2011), indirectly inhibits proteasome activity by inhibition of dual-specificity tyrosine-regulated kinase 2 (Banerjee et al., 2018) and also blocks COP signalosome-associated kinases (Uhle et al., 2003). It should be noted, however, that natural products such as curcumin have been associated with extensive polypharmacology (Baell, 2016; Gupta et al., 2011; Hatcher et al., 2008; Kasi et al., 2016; Nelson et al., 2017).

Modest levels of polyubiquitination were observed in cells exposed to BCI–Cl, an inhibitor of dual-specificity phosphatase 6 (Dusp6) (Molina et al., 2009). Polyubiquitinated proteins accumulating in BCI–Cl-exposed cells were found to be associated with proteasomes (Fig. 6), strengthening the notion of proteasome inhibition. Furthermore, BCI–Cl inhibited degradation of the Ub266–YFP reporter protein. BCI–Cl ranked high in the CMap analysis and also displayed a pattern of sensitivity in the NCI60 panel (COMPARE analysis) that strongly correlated to that of the proteasome inhibitor bortezomib. These findings all suggest that proteasome inhibition is either a direct, or possibly indirect, mode of action of this compound. BCI–Cl was reported inhibit the proliferation of gastric cancer cells both in vitro and in animal models (Ramkisson et al., 2019; Wu et al., 2018) and it is conceivable that these antineoplastic effects may partially be due to UPS inhibition and possibly not to Dusp6 inhibition.

Manumycin A also induced increases in polyubiquitinated proteins in exposed cells. This natural product (from Streptomyces parvalus) has been described to inhibit farnesyltransferase activity (Hara et al., 1993) and protein phosphatase 1 alpha (Carey et al., 2015) but has also been described to increase cellular levels of polyubiquitinated proteins (Singha et al., 2013). More recently, manumycin A was shown to inhibit thioreredox reductase (Tuludhar and Rein, 2018), consistent with the electrophilic properties of the compound reacting with the nucelophilic Sec residue of this enzyme. The gold compound auranofin also induced modest accumulation of polyubiquitinated proteins and stabilization of the Ub376–YFP reporter. Similar to manumycin A, auranofin is an inhibitor of thioreredox reductase and has been associated with induction of oxidative stress and tumor cell apoptosis (Gandin et al., 2010). Auranofin has, however, also been described to inhibit proteasome-associated DUBs when used at high concentrations (Liu et al., 2014; Zhang et al., 2019). Withaferin A, a natural product from Withania somnifera (Indian Winter cherry), was also found to induce increases in cellular polyubiquitin and stabilization of the Ub376–YFP reporter in cells. Withaferin A was originally described as an inhibitor of the proteasome (Yang et al., 2007) but has later been reported to target p97/VCP (Tao et al., 2015). p97 VCP is involved in the control of protein homeostasis by extracting ubiquitin-modified proteins from the endoplasmic reticulum for delivery to the proteasome (Jentsch and Rumpf, 2007).

The ranking of compounds using CMap and COMPARE resulted in quite different results. This was expected since CMap analyses will be performed at concentrations that will generate cellular responses whereas the results of COMPARE analyses will be affected both by mechanisms of action and parameters such as drug uptake and metabolism (that will affect drug sensitivity). Thus, two compounds having identical mechanisms of action will generate similar gene expression profiles but may not necessarily display the same sensitivity patterns if one, but not the other, is a substrate for a drug exporter. Parthenolide, a natural product from the feverfew plant, ranked highest in the COMPARE analysis but did not score high in the CMap analysis. Parthenolide exerts multiple biological activities including anti-cancer effects (Pei et al., 2013). Parthenolide was recently demonstrated to covalently modify cysteine 427 of focal adhesion kinase 1, leading to impairment of downstream signaling pathways (Berdan et al., 2019). Parthenolide has also been shown to inhibit the DUB USP7 (Li et al., 2020) and to target Hsp72 (Shin et al., 2017), showing multiple mechanisms of action. Whereas parthenolide treatment did not lead to increased levels of polyubiquitin in cells, parthenolide increased the expression of the chaperone Hsp70B*. This effect was observed for almost all compounds tested and was most pronounced in cells exposed to 17-DMAG, an analogue of the natural product geldanamycin, and radicicol. 17-DMAG and radicicol are inhibitors of Hsp90 (Blagowski, 2002; Porter et al., 2009) and have previously been shown to induce Hsp70 expression (Madrigal-Maute et al., 2010). The general stimulatory effect on Hsp70 expression by various compounds tested is a likely reason for the generation of gene expression profiles that are related to those induced by proteasome inhibitors. An alternative, or contributing, mechanism for induction of Hsp70 expression is a direct effect on the heat shock factor (HSF) transcription factor. Electrophilic compounds are known to covalent modify thiols in HSF resulting in activation and stimulation of the expression of chaperone genes (Cajone and Crescente, 1992; Liu et al., 1996). Interestingly, a screen for small molecules inducing a reporter driven by the heat shock response element (HSE) identified a number of natural products containing Michael acceptors (Sanjagata et al., 2011). This finding was interpreted in terms of natural products being selected during evolution to elicit this response but could represent a direct effect of these compounds on the HSF. An additional factor that could potentially contribute to the induction of chaperones is
compound hydrophobicity that could result in non-specific protein modifications and mild proteotoxic stress (Ohnishi et al., 2013).

Eleven Michael acceptor compounds that have been demonstrated to inhibit proteasome activity in cells (Selvaraju et al., 2019) are displayed in Fig. 7. Despite being classified as both PAINS and Badapples, accumulation of the UbD76-YPF reporter induced by five of these compounds showed a modest/strong association to cell death. This finding raises the possibility that Michael acceptor-containing compounds can elicit an apparently defined pharmacological response. If so, one explanation would be that although electrophiles will interact with multiple targets, these targets will not be of equivalent importance for tumor cell viability/proliferation. Functional genetic screens have shown that approximately ninety percent of cellular genes are non-essential for proliferation of tumor cells in vitro (Wang et al., 2015). Small molecules that bind to such non-essential proteins will not be effective in cell proliferation assays. The proteasome is, however, together with the spliceosome and ribosome, essential for the viability of tumor cells (Nijhawan et al., 2012).

Michael acceptors are considered as unattractive by drug developers. However, it is important to make the distinction between compounds that could potentially be developed into drugs and compounds that can be used as chemical probes. Compounds containing α,ω-unsaturated carbonyls are unlikely to be useful as tool compounds due to their reactivity. They are, however, not necessarily disqualified as potential anticancer drugs since drugs simply need to be safe and efficacious.

CRediT authorship contribution statement

Arjan Mofers: performed laboratory work, bioinformatics, Writing - original draft. Karthik Selvaraju: performed laboratory work, Writing - original draft. Johannes Gubat: bioinformatics, Writing - original draft. Padraig D’Arcy: Supervision, provided funds, Writing - original draft. Stig Linder: performed bioinformatics, Supervision, provided funds, Writing - original draft.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2020.173709.

References


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