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Novel Protein Disulfide Isomerase Inhibitor with Anticancer Activity in Multiple Myeloma


Introduction

Despite progress, multiple myeloma remains largely incurable, recruiting relapses from genetically heterogeneous multiple myeloma subclones (1, 2). Cytopenias from cumulative treatment toxicity and refractory multiple myeloma limit therapeutic options and lifespan creating need for bone marrow sparing approaches effective against genetically highly variable myeloma cell populations.

Defense against stress misfolded proteins exert the endoplasmic reticulum (ER) has been identified as Achilles heel of multiple myeloma (3). Proteins destined for secretion enter the ER in an incompletely folded state and factors commonly found in cancer, like augmented protein synthesis, nutrient deficiency, and hypoxia increase them or impair their folding exerting ER stress (4). In multiple myeloma, high protein synthesis related to neoplastic behavior is amplified by plasma cell program activation yielding the highest protein secretion rate known in mammalian biology (5). Furthermore, secreted antibodies have complex folding needs involving correct arrangement of multiple intra- and intermolecular disulfide bonds (5, 6). If misfolded proteins accumulate in the ER gradually induction of the transcription factor CHOP (C/EBP homologous protein) triggers suicide (4) whereas acute misfolding events like severe hypoxia cause apoptosis via opening of ER calcium channels (7). One way cells attempt to evade such fate is by transport of misfolded ER proteins into the cytoplasm for ubiquitination and proteasomal degradation (6, 9). Interference with disposal of naturally occurring misfolded antibodies appears critical for clinical success of the proteasome inhibitor bortezomib in multiple myeloma (10, 11), suggesting a blockade of the folding mechanism could further improve multiple myeloma therapy. Only the family of protein disulfide isomerases (PDI), which contains 17 members with 1–4 active CXXC or CXXC-like motifs (12), can accomplish correct arrangement of disulfide bonds in the ER through reductase, oxidase, and isomerase functions.

To streamline selection of anti–multiple myeloma drug candidates for further study, we chose a mechanistically unbiased approach and filtered compounds by assessment in one
multilayered assay modeling major barriers to clinical success; liver metabolism, need for diffusion, transient exposure, myeloma-supporting niche, and bone marrow toxicity. The lead compound (CCF642) had broad anti–multiple myeloma activity, was effective in vivo, and appeared to act, at least in part, by inhibition of PDI through a novel mechanism. Here we describe the discovery and initial characterization of CCF642.

### Materials and Methods

#### Cells

RPMI 6226, NCI-H929, MM1.S MM1.R, U266, Jeko-1, Rec-1, Mino, JVM-2, SU-DHL-6, SU-DHL-16, K562, KG-1, GDM-1, and HS-5 were from ATCC (authenticated there by STR). KMS-12-PD and KMS-12-IHM were from ICRB (authenticated there by STR) and JIN-3 from DSABZ (authenticated there by multiplex PCR of minisatellite markers). STGML-luc mouse multiple myeloma cells were kindly provided by Dr. Babatunde O. Oyajobi, University of Texas Health Science Center at San Antonio (San Antonio, TX), where this cell line was discovered and characterized (13–16). All of the above cells were kept in culture for less than 6 months including expansion for aliquot freezing. HRMM.09 cells were established from blood of a patient with secondary plasma cell leukemia under Institutional Review Board (IRB)-approved consent in our laboratory. MM1.S-luc, HRMM.09-luc, and NCI-H292-luc were transduced with firefly luciferase lentivirus (Qiagen), not further authenticated, but used within 6 months after luciferase expression confirmation. Anti-CD138 magnetic bead-purified peripheral blood plasma cells from two patients with secondary plasma cell leukemia (sPCL-1, -2) who consented to an IRB-approved sample collection protocol were used after 4 weeks in culture and one Ficoll density centrifugation to remove dead cells. Normal bone marrow mononuclear (NLBM) cells were from discarded bags of bone marrow grafts from healthy stem cell donors under an IRB-approved consent waiver. Human multiple myeloma cells were grown in RPMI 1640, STGML-luc in IMDM, HS-5 in DMEM, and NLBM cells in RPMI supplemented with 2% (v/v) of supernatant removed from confluent HS-5 cell cultures; all media was supplemented with 10% FBS, penicillin G (50 units/ml), and streptomycin (50 μg/ml). All cells cultured at 37°C, 5% CO2 in humidified air.

#### Primary screen

A total of 30,335 chemically diverse small molecules were screened for anti–multiple myeloma activity in MM1.S cells using a single-step cellular ATP quantitation assay (Supplementary Table S1 and Supplementary Methods).

#### Multilayered (sandwich) in vivo model

HS-5 bone marrow stromal cells were grown to confluence on semipermeable bottoms of 24-well Transwell inserts (Corning), then MM1 S-luc cells were added and cocultured overnight. The next day, drug candidate was suspended into C57BL/KaRij mouse liver homogenate (preparation in Supplementary Methods), incubated at 37°C for 30 minutes, then mixed with 35–40°C warm low gelling temperature liquid agarose (Sigma-Aldrich), and immediately dispensed into 24-well plates where it was allowed to solidify at room temperature for 30 minutes. NLBM cells were layered on top and left there, whereas HS-5/MM1.S-luc cell containing inserts were moved after 1 to 3 hours with about 25% of the drug-equilibrated media in them into drug-free liver/agarose/media wells to decrease concentrations of drugs by estimated 75% once a new equilibrium is reached, assuming no liver metabolism occurs. Three to 4 days later, multiple myeloma luciferase activity was measured after addition of firefly α-luciferin, 0.5 mg/ml (Gold Biotechnology), and viability of aspirated NLBM cells was assessed by trypan blue exclusion using Vicell flow (Beckman Coulter).

#### Medicinal chemistry

CCF642, biotinylated CCF642 (B-CCF642), and CCF642-COOH were synthesized in our laboratories. Synthetic schemes and graphs confirming their identity by 1H-NMR and mass spectrometry are in Supplementary Methods and Fig. S1, respectively.

#### PDI activity

PDI activity was measured according to a published protocol using di-rosin-diglutathione (di-E-GSSG) as pseudo substrate for sensitive assessment of PDI’s reductase activity (17) with minor modifications and details in Supplementary Methods.

#### Computational modeling and molecular dynamics

The computational model used the reduced PDI (NP_000909.2) crystal structure (18), as binding of biotinylated CCF642 to PDI was enhanced after reduction with dithiothreitol (DTT). Docking of CCF642 on PDI (PDB ID: 4EKZ) used Autodock 4.1 (In Autodock Tools 1.5.2). Binding energy calculations used CHARMM force field in Discovery Studio 1.3 pipeline (Accelrys, Inc.). Final image processing and electrostatic surface potential calculation used PyMOL (www.pymol.org).

#### Single-cell cytoplasmic calcium measurements

MM1.S cells were loaded with Fura 2-AM and dynamic changes in cytoplasmic calcium concentration were measured by single-cell digital imaging as described previously in detail (19).

#### PDIA1 site-directed mutagenesis

Human cDNA clone NM_000918 (Origene) was subcloned from pCMV-6- XL4 into pT7CE1-CHis (Thermo Fisher Scientific) for site-directed mutagenesis (primer sequences in Supplementary Methods) using QuickChange II XL (Agilent Technologies). Proteins were purified with His-tagged purification miniprep-kit (Clontech).

#### Protein S-nitrosylation

Protein S-nitrosylation was investigated via biotin switch method using Cayman Chemical kit (cat # 10006518) according to manufacturer’s instructions.

#### Genotoxicity screen

Genotoxicity screen used SOS chromotest kit with S9 rat liver enzymes (ebpi) according to manufacturer’s instructions.

#### Animal experiments

Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH, and conducted under an approved Institutional Animal Care and Use Committee protocol. C57Bl/KaRij mice (Harlan Laboratories) of 6 to 8 weeks of age were injected with STGML-luc cells via tail vein the day before drug treatments were started. CCF642 was administered as albumin formulation intraperitoneally,
bortezomib in PBS subcutaneously. Life imaging using Xenogen IVIS CCD was performed 7 minutes after subcutaneous injection of luciferin (Gold Biotechnology). Statistical analysis used JMP 10.0.X software (SAS Institute, Inc.). Retro-orbital plexus blood draws were done after isoflurane (3%) inhalation anesthesia and complete blood counts obtained on Hemavet 950FS (Drew Scientific), which gives mouse reference values; plasma biochemical tests were analyzed on cobas c311 (Roche Diagnostics) with gender specific biochemical reference values from C57Bl/6j mice (20) as reference.

Results

Mechanistically unbiased discovery of anti-myeloma compound CCF642

To identify new clinically translatable treatments for the incurable cancer multiple myeloma that disrupt critical programs for this malignancy while sparing normal bone marrow, we used a mechanistically unbiased algorithm on a library of 30,335 small molecules (Fig. 1). The first step tested for anti–multiple myeloma activity. It identified 225 compounds that suppressed cellular ATP in MM1.S cells to ≤ 30% of vehicle control at ≤ 5 μmol/L after 3 to 4 days (Fig. 1 and Supplementary Table S1). Step two sought to filter these 225 agents for clinical promise in terms of relative tolerance by NLBM and ability to overcome pharmacokinetic and pharmacodynamic barriers to anti–multiple myeloma activity. Emitted from agarose-embedded liver homogenate that was able to reduce the activity of hepatically metabolized bortezomib (Supplementary Fig. S2); only three drug candidates demonstrated promising anti–multiple myeloma activity in a setup that included resistance conferring HS-5 bone marrow stroma cells (21) as niche and a method to decrease drug concentrations through non-hepatic means after 90 minutes by transfer of MM1.S-luc/HS-5 containing semipermeable inserts into drug-free liver/agarose/ media containing wells. None of the three hits inhibited the number of surviving NLBM cells by more than 30%, although they were exposed to drug-emitting liver constantly for 4 days (Figs 1 and 2B). Bortezomib, known to be relatively well tolerated by normal bone marrow at doses needed to control multiple myeloma, had a similar therapeutic window in this assay (Fig 2B).

In the third step, we tested whether compounds targeted programs of general importance in multiple myeloma via cytotoxicity assessment in a cell line panel. All three hits of step 2 had sub- or low micromolar IC_{50} (<5 μmol/L) against all of at least seven genetically heterogeneous multiple myeloma cell lines tested per compound using trypan blue flow cytometry with constant exposure over 72 hours (Fig. 2C). CCF642 was most potent with submicromolar IC_{50} in 10 of 10 multiple myeloma cell lines tested (Fig. 2C).

Step four asked whether the in vivo model indeed yielded molecules with in vitro activity. To this end, we used an aggressive syngeneic mouse model of myeloma (15) that causes typical myeloma bone disease and death of C57Bl/6j mice after 1 to 2 months after tail vein injection of luciferase expressing STG1M1 cells (STG1M1-luc). STG1M1-luc mouse multiple myeloma cells were included in the otherwise human multiple myeloma cell line panel (Fig. 2C) to ensure the target was expressed in mice as well. Limited solubility could be addressed by dissolving DMSO stock of CCF642 and CCF1172 into albumin solution, which had no toxicity as vehicle on multiple myeloma cells and did not affect potency of hits against multiple myeloma cells in vitro, but the best solution we identified for CCF1118, 0.8% Tween-20 in sterile water, did not allow dosing above 4 mg/kg. With these limitations in mind, CCF642 at 10 mg/kg intraperitoneally three times a week yielded most convincing therapeutic effects, significantly prolonging life of STG1M1-luc-bearing mice and suppressing STG1M1-luc growth as determined by life imaging (Fig 2D). We therefore concentrated on CCF642 and initially evaluated cytotoxicity against other hematologic malignancies, which revealed similar IC_{50} in osteogenically related lymphoma, whereas a three-cell line screening sample of myeloid neoplasia showed more varied responsiveness. Primary cells from patients with refractory myeloma who developed secondary plasma cell leukemia (sPCL) also had IC_{50} below 1 μmol/L, whereas three separate NLBM samples confirmed NLBM tolerance up to 6.75 μmol/L, the maximal dose tested (Supplementary Fig. S3).

Next, we compared CCF642 to the most potent FDA-approved upfront drug for myeloma, bortezomib. CCF642 at 10 mg/kg intraperitoneally three times a week significantly improved lifespan of mice compared with albumin vehicle. Bortezomib given at the MTD for this mouse strain (0.6 mg/kg subcutaneously twice a week) yielded similar prolongation of life, not statistically different from CCF642 (P = 0.18, log-rank test), and life imaging on day 23 documented comparable significant suppression of STG1M1-luc growth. Treatments appeared to be well-tolerated on the basis of normal behavior and stable weights (Fig. 3A–C). To
explore safety further, blood after 15 and 28 days of treatment from five additional STG1M1-luc-bearing mice per time point was obtained to screen for toxicity on bone marrow (blood counts), liver (alanine aminotransferase, ALT), kidney (creatinine), and effects on glucose metabolism. Except for asymptomatic increase of neutrophils, parameters remained within reference range after CCF642 (Fig. 3D). Culture of STG1M1-luc and MM1.S-luc cells in bortezomib-containing media over 6 months increased the IC50 toward this drug but not toward CCF642 in both cell lines, suggesting CCF642 may prove useful in the context of bortezomib resistance and acts via a different mechanism (Fig. 4).

An active biotinylated analogue identifies PDI as CCF642 target

To identify the target of CCF642, we introduced a biotin moiety at its 4-methoxy group (Fig. 5A) expecting this was not the target interaction site since a commercially available unsubstituted phenyl analog retained anti–multiple myeloma activity, although IC50 in MM1.S was increased from 0.25 to 1.8 μmol/L. Biotinylated CCF642 (B-CCF642) also had higher IC50 (2.9 μmol/L), but at 6.75 μmol/L, MM1.S survival was comparable to CCF642 (~20% at 72 hours), suggesting it may act intracellularly but enter less well. Streptavidin immunohistochemistry confirmed entry into the cytoplasm of MM1.S (Fig. 5B). Treatment of MM1.S cells with B-CCF642 followed by SDS-PAGE and biotin staining suggested covalent interactions are formed with a main band at around 60 kDa (Supplementary Fig. S4). Mass spectrometry of streptavidin-bead purified protein lysates from B-CCF642–treated and untreated MM1.S cells run through SDS-PAGE and cut from a corresponding area of the gel (50–70 kDa) revealed protein disulfide isomerase A1 (PDI-A1, protein disulfide isomerase precursor) as most abundant protein found only after treatment. Two other PDI family members, PDI-A3 (protein disulfide isomerase precursor A3) and PDI-A4 (protein disulfide isomerase precursor A4), were also only identified in treated samples (Supplementary Table S2).

Incubation of recombinant PDI with B-CCF642 with or without prior CCF642 incubation followed by SDS-PAGE and biotin stain confirmed PDI as common target (Fig. 5C). B-CCF642 also covalently bound to recombinant PDI-A3 but not to BSA used in the animal treatment formulation for its solubility-enhancing properties (Fig. 5C). To test whether CCF642 affects PDI function, we performed di-E-GSSG assays, which measure PDI’s reducing capacity (17), and compared inhibition to known PDI inhibitors PACMA 31 (22) and LOC14 (23). CCF642 at 1 μmol/L inhibited reduction of di-E-GSSG about as much as 100 μmol/L of LOC14 or PACMA 31 (Fig. 5D). Accordingly, the anti–multiple myeloma activity against three exemplary multiple myeloma cell lines was most pronounced with CCF642 (Fig. 5E). These observations raised the question of how CCF642 inhibits PDI.

CCF642–PDI binding model

Computational docking argued against covalent binding to active site cysteine used by PACMA 31 and all other known covalently binding PDI inhibitors (22, 24), as CCF642 could not be approximated any closer than 5 Ångström. The amino group of adjacent conserved functionally relevant lysine (25), with 2.8 Ångström came close enough to the carbonyl group of CCF642.

Figure 2.
Results of anti–multiple myeloma screening algorithm for the three hits. A, hit structures. B, viable cells at 96 hours in the in vivo model, with 90-minute exposure of the MM1.S-luc/HS5 insert to small molecule–emitting liver at calculated maximal peak concentrations of 15 μmol/L for hits, 30 μmol/L for bortezomib. C, reduction of trypan blue-negative, viable cells by 50% compared with vehicle control (IC50) in a panel of genetically heterogeneous multiple myeloma cell lines treated continuously with hits for 72 hours. ND, not done. D, in vivo activity of CCF642 in the aggressive syngeneic STG1M1-luc/KaLu WR mouse model of myeloma as determined by life imaging (top) and statistically significant (log-rank test) extension of survival (bottom). In vivo results display at least two independent experiments with at least duplicates. Error bars, SD.
to propose a covalent reaction (Fig. 5F). B-CCF642 binding studies on PDIA1 mutants targeting both active sites were consistent with this computational hypothesis. Replacement of lysine with glutamic acid completely blocked, whereas the NH$_2$ side chain containing glutamine allowed weak binding; replacement of cysteine with serine had no effect (Fig. 4G). Definitive crystallographic binding studies are beyond the scope of this article but clearly needed since a covalent reaction with lysine would likely require its deprotonation and it is not known whether this occurs during PDI’s redox reactions or upon entry of CCF642 into its enzymatic pocket. Computationally, additional non-covalent interactions were predicted between NO$_2$ of the nitrothiophene moiety of CCF642 and the NH$_2$ side chain of glutamine adjacent to lysine (Q402 in Fig. 5F). All PDI family members pulled out from multiple myeloma cells with B-CCF642 have NH$_2$ side chain containing amino acids at that site, suggesting they may be important for PDI activity and for binding of B-CCF642. Accordingly, substitution of the NO$_2$ group in CCF642 with COOH reduced anti-PDI activity (Fig. 6A) and binding to PDI as assessed by competition with B-CCF642 (Fig. 6B). To investigate whether the NO$_2$ group might be released from CCF642 and inhibit PDI via S-nitrosylation, we incubated recombinant PDI with CCF642 or S-nitrosothiol and tested for nitrosylated PDI using the biotin switch method (26). We found no evidence for S-nitrosylation of PDI with CCF642, whereas the positive control effectively caused this change (Fig. 6C). Furthermore, the bacterial SOS chromotest found no evidence for genotoxicity of CCF642 up to 20 μmol/L, with or without prior liver enzyme incubation (Fig. 6D), arguing against nonspecific DNA toxicity, which could occur with NO$_2$ release or other DNA toxic effects. The reaction between CCF642 and CGHCK motifs of PDI family members therefore likely leaves CCF642 intact. The putatively novel mechanism
PDI inhibition, higher potency than known PDI inhibitors, and favorable safety signals from NLBM, animal studies, and the bacterial genotoxicity screen warranted pursing the mechanism of antimalma action of CCF642 further.

Acute ER stress with apoptosis-inducing calcium release in myeloma by CCF642
Treatmen of myeloma cells with CCF642, PACMA 31, or LOC14 at doses in the upper clinically achievable range leads to accumulation of misfolded proteins in the ER as documented by dimerization of PERK via phosphorylation (3, 4) and oligomerization of IRE-1α (27), most pronounced after CCF642, but suggesting they may all be able to inhibit PDI in vivo (Fig. 7A). Increase of the proapoptotic unfolded protein response (UPR) mediator CHOP is evident within 30 minutes for all compounds but interestingly XBP1-s, a critical transcription factor for mediating the adaptive UPR (3, 4), undergoes a time-dependent decline that correlates with PARP cleavage, suggesting it may be degraded by apoptotic enzymes along with misfolded protein sensors PERK and IRE-1α (Fig. 7A). To investigate whether XBP1 activation via unconventional splicing of its cytoplasmic mRNA by dimerized IRE-1α is intact we performed RT-PCR (Fig. 7B). In these experiments, MM1.S cells were used, as it was known that despite baseline ER stress, spliced XBP1 mRNA is not detectable in them without exogenous stressors (28). CCF642 caused IRE1-α dimerization within 15 minutes, peaking at 1 hour, and within 2 hours, spliced XBP1 mRNA could be detected confirming IRE1-α dimers were functionally intact (Fig. 7B) and followed a similar time course as the other main ER stress sensor, PERK (Fig. 7C). Nuclear fractions demonstrated decrease of the transcription factor XBP-1s within 15 minutes compromising defense against ER stress, probably due to acute induction of apoptotic pathways by CCF642 (Fig. 7A and E). As kinetics of apoptotic pathway induction were faster than predicted for the transcription factor CHOP, we hypothesized acute increase of misfolded proteins might trigger apoptosis-inducing release of ER calcium stores. Indeed, single-cell cytotoxic calcium measurements in MM1.S and NLBM after 3 μmol/L CCF642 revealed selective increase in MM1.S that followed a similar time course as ER stress sensors (PERK, IRE1-α) and apoptotic pathway kinetics, whereas NLBM did not respond noticeably with ER stress sensors (total PERK and IRE-1α antibodies yielded no signals, shown PERK is faint) and minimally cleaved caspase-3 (Fig. 7B–D and F). BAPTA AM, a cell-permeable calcium chelator dose, dependently delayed caspase-3 and PARP cleavage in MM1.S by CCF642 (Fig. 7E), suggesting calcium release contributed to apoptosis.

Discussion
Here we report the discovery of a novel antimalma drug candidate with inhibitory function on the only enzyme family capable of folding disulfide-rich secreted proteins like antibodies through a mechanistically unbiased screening algorithm, which simulated key barriers to in vivo success.

Efficacy of lead CCF642 in a syngeneic mouse myeloma model (Figs. 2 and 3) comparable to fully optimized FDA-approved first-line proteasome inhibitor bortezomib (Fig. 3) confirms that an anti–multiple myeloma assay with highly simplified modeling of liver, non-hepatic clearance, and bone marrow (Figs. 1 and 2B) can identify promising drug candidates. CCF642 was one of three structurally distinct compounds from a 30,335 small-molecule screening algorithm demonstrating desired anti–multiple myeloma activity without substantial bone marrow toxicity in this assay that we applied as low-throughput manual screen on 225 confirmed active anti-multiple myeloma small molecules. The two other compounds (Fig. 2) were less convincing during pilot in vivo experiments, but this may in part be related to difficulties with their formulation (CCF1118) or lower potency (CCF1172). The described setup with lymphoid and myeloid growth factor secreting HS-5 bone marrow stromal cells as niche, documented to confer resistance not only to multiple myeloma (21, 29) but also to myeloid and other lymphoid cells (30–32), should be applicable to a variety of bone marrow cancers but would require adjustment for solid tumors. Compared with reported cancer-niche screens (33), growth in semipermeable inserts expands modeling options like simulation of non-hepatic clearance without disruption of cell contacts by transfer into drug-free media. Use of inserts further enables compartmentalized NLBM growth and inclusion of liver homogenate. Applied to chemical library compounds it will not mimic in vivo pharmacokinetic behavior but has the potential to enrich for agents with higher likelihood of in vivo activity by filtering for stability toward liver enzymes, ability to diffuse (through agarose and cell layers), and suppress cancer on niche with adjustable bolus-like or constant exposure. Our results suggest it may further enable selection of agents without substantial adverse effects on the bone marrow (Fig. 3D). If others confirm our findings and automation is achieved, it may
Figure 5.
PDI is a target of CCF642. A, biotinylated CCF642 (B-CCF642). B, streptavidin IHC confirms entry of B-CCF642 into the cytoplasm of MM1.S cells. C, CCF642 and B-CCF642 compete for binding to recombinant PDIA1, quantified by densitometry of immunoblot images normalized to background and PDI AB signals (left). The right immunoblot panel shows no evidence for covalent B-CCF642 binding to BSA after 30-minute incubation but to PDIA3. Ponceau S stain was used to document protein loading. D, time-dependent inhibition of di-E-GSSG reduction by PDI with CCF642 or known PDI inhibitors PACMA and LOC14 normalized to spontaneous di-E-GSSG reduction. E, reduction of trypan blue-negative cells at 72 hours in three multiple myeloma cell lines by CCF642 and known PDI inhibitors at up to 6.75 μmol/L. F, electrostatic surface representation of CCF642 docked onto PDI (Protein Data Bank ID: 4EKZ), as predicted by Autodock 4. Energy minimization suggested covalent binding may occur to conserved lysine K401, where the carboxyl group of CCF642 is predicted to reach within 2.8 Å of its amino group, whereas distances of 4.58 and 5.83 Å between the oxygen atoms of the NO2 group of CCF642 and the NH group of glutamine 402 suggest noncovalent interaction could occur at this site. A similar situation is predicted for the other active site CGHEK motif and for other PDIs sharing this motif. G, biotin stain and anti-His AB immunoblot after a 30-minute incubation with 5 μmol/L B-CCF642 of recombinant wild-type PDIA1 and PDI mutants that have both active sites (a and a') mutated as indicated shows B-CCF642 binding is dependent on lysine but not on cysteine. All experimental results under this figure are representative of at least three independent experiments. Error bars, SD.
streamline cancer drug discovery by reducing unsuccessful costly animal experiments.

Like many small molecules from diversity libraries, CCF642 has limited aqueous solubility, creating challenges when moving from in vitro to in vivo where higher volume of distribution requires administration of more concentrated drug. After standard solvents used for intravenous formulations failed to keep CCF642 in solution, we explored albumin, an abundant plasma protein with hydrophobic and hydrophilic surfaces. Albumin is FDA-approved as carrier for paclitaxel in Abraxane and bovine as well as human albumin has been used in mice effectively for drug delivery (34, 35). Albumin-dissolved CCF642 could be given intravenously without injection reactions in mice, but for ease of administration, we chose the intraperitoneal route and saw promising efficacy without obvious toxicity. Albumin could generally be considered for clinical translation but we anticipate medicinal chemistry optimization will enable a less expensive formulation. Respective efforts will aim for crystallographic confirmation of the putatively novel interaction with PDI (Fig. S5 and G) to allow rational design and analog pharmacophore testing. The thiazolidinedione-like structure of CCF6-42 and its ability to increase cytosolic calcium in myeloma cells suggest special attention will need to be placed on liver, mitochondrial, and cardiac safety in the analog selection process. Preliminary and limited safety analyses of CCF642 in myeloma-bearing mice found no evidence for liver toxicity or fluid accumulation anti-diabetic PPAR-activating thiazolidinediones can cause, but mild decrease of blood sugar (Fig. 3D) reinforces the need for vigilance and suggests a possible off-target effect of CCF642 will need to be investigated. Stable cytosolic calcium in NLBM cells after CCF642 (Fig. 7D) argues against broad nonspecific mitochondrial toxicity but does not obviate the need for further investigation into possible effects of optimized analogs on liver or heart. Furthermore, asymptomatic increase of neutrophils in intraperitoneal albumin-CCF642–treated mice (Fig. 3D) suggests optimizing formulation to avoid delayed precipitation that may have subclinically irritated peritoneal membranes and investigating possible alternate mechanisms will be important in drug development. Although the CCF642 scaffold raises outlined safety concerns, our preliminary analyses suggest it may yield an optimized analog that satisfies safety mandates for clinical translation.

Success of the proteasome inhibitor bortezomib has established protein homeostasis as valid target in oncology, especially for lymphoid malignancies (11, 36) but multiple myeloma remains incurable costing estimated 11,240 lives in 2015 in the United States (SEER). CCF642, consistent with its effect on proteostasis by PDI inhibition (Figs. 5 and 7), had potent activity in the submicromolar IC₅₀ range on multiple myeloma and lymphoma cell lines while so far limited evaluations in myeloid neoplasia revealed more varied and less pronounced effects.

Figure 6. The NO₂ group of CCF642 is important for PDI inhibition but does not lead to S-nitrosylation or genotoxicity. A, structure of CCF642 analog with COOH instead of NO₂ (top), which is about 10x less potent than parent CCF642 in the di-E-GSSG PDI activity assay (bottom). B, CCF642-COOH competes with B-CCF642 for PDI binding but less potently than CCF642 by densitometric analysis (Fig. 5C). C, PDI was incubated with 10 µmol/L CCF642 or 10 µmol/L S-nitrosoglutathione for 1 hour before processing according to the boitin switch method that exchanges S-nitrosylated sulfhydryl groups with boitin. D, SOS chromotest bacteria (ebsp, Inc.) induce β-galactosidase in response to genotoxic stress and constitutively express alkaline phosphatase. The graph shows increase in β-galactosidase/ alkaline phosphatase signals = SOS activity after 2-hour incubation at indicated doses for the genotoxic agent 4NQO and for 2AA, which becomes genotoxic after liver enzyme incubation. CCF642, with or without liver incubation, did not increase SOS activity over untreated control. Results are representative of three independent experiments. Error bars, SD.
Acute ER stress with apoptosis-inducing calcium release in myeloma by CCF642. A, PDI inhibitors increase PERK dimerization by phosphorylation and IRE1-α oligomerization within 30 minutes in KMS-12-PE confirming accumulation of misfolded ER proteins. Proapoptotic ER stress signaling via CHOP induction is intact with all PDI inhibitors but XBP-1s, a transcription factor mediating adaptive ER stress responses, decreases in a temporal pattern that correlates with PARP cleavage, suggesting breakdown along with PERK and IRE1-α. PDI staining documents comparable loading. B, Immunoblots and RT-PCR of MM1.S whole cell, nuclear, and cytoplasmic fractions show XBP1 splicing (s) of cytoplasmic unspliced (u) mRNA in response to CCF642 is intact and detectable about 1 hour after dimerization of IRE1-α peaks. Nuclear XBP1-s protein undergoes a decline that correlates with PARP cleavage (E) in this cell line as well. C, PERK dimers in MM1.S cells treated with CCF642 confirm increase of misfolded proteins in the ER of MM1.S cells after 15 minutes. D, MM1.S and NLBM cells were loaded with the intracellular calcium indicator Fura-2 AM, and cytoplasmic calcium concentration was continuously recorded following addition (arrow) of 3 μmol/L CCF642 or DMSO vehicle. Each trace is average calcium concentration in 86 to 89 cells. E, Immunoblots show that pretreatment of MM1.S cells with intracellular calcium chelator BAPTA AM for 1 hour before 3 μmol/L CCF642 delayed caspase-3 and PARP cleavage. F, Immunoblot of NLBM cells after CCF642 treatment reveals minimal increase in PERK dimers and minimal caspase 3 cleavage. Results are representative of at least three independent experiments per panel.
Calcium-regulating enzymes, like Ero-1α (37), that restore oxidative potential of PDI required for immunoglobulin folding (5). Therefore, current in vitro PDI activity assays mainly establish that a drug candidate can affect PDI function but not how effectively its versatile enzymatic potential is impaired in vivo or how profoundly restoration by Ero-1α is affected. We chose the most sensitive in vitro PDI activity assay to quantify inhibition and then evaluated whether the most upstream expected consequence of PDI inhibition occurred in cells, sensing of misfolded ER proteins by PERK and IRE1-α. CCF642 was more potent than known PDI inhibitors in the in vitro assay against myeloma cells (Fig. 5) and when assessed for ability to increase misfolded ER proteins in multiple myeloma cells, but the latter experiments that used doses in the upper range of generally clinically achievable (3 μM/L) suggested that all PDI inhibitors may reach their target in vivo (Fig. 7A). For CCF642, the only PDI inhibitor where this dose was above the IC₅₀ against multiple myeloma cells and against PDI in vitro, this was followed by acute induction of apoptosis, faster than expected from induction of the proapoptotic ER stress mediator CHOP. Further studies suggested that calcium release, possibly from rapid accumulation of misfolded ER proteins, was at least partially responsible (Fig. 7). Detailed studies on the mechanism of calcium release by CCF642 in multiple myeloma are beyond the scope of this article but might reveal additional targets or interference with interaction of PDI with calcium-regulating proteins like calreticulin (38).

In summary, results suggest a straightforward assay that simulates important barriers to in vivo success of anti–multiple myeloma compounds can yield candidates promising for clinical translation. While its first lead may act through mechanisms in addition to PDI inhibition, it is so far the most promising anti–multiple myeloma PDI-inhibiting compound. Fully defining its interaction with PDI through crystallography may enable development of optimized specific PDI inhibitors with potentially broader oncology application.

Disclosure of Potential Conflicts of Interest
S. Vatolin, J.G. Phillips, D. Grabowski and F.J. Reu have ownership interests and filed a patent application for CCF642 and a provisional patent application for CCF1172. S. Vatolin and F.J. Reu have ownership interests and filed patent application for the three organ system assay. No potential conflicts of interest were disclosed by the other authors.

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