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Curcusone D, a novel ubiquitin–proteasome pathway inhibitor via ROS-induced DUB inhibition, is synergistic with bortezomib against multiple myeloma cell growth

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ABSTRACT

Background: Ubiquitin–proteasome pathway (UPP) plays a very important role in the degradation of proteins. Finding novel UPP inhibitors is a promising strategy for treating multiple myeloma (MM).
Methods: Ub-YFP reporter assays were used as cellular UPP models. MM cell growth, apoptosis and overall death were evaluated with the MTS assay, Annexin V/PI dual-staining flow cytometry, poly (ADP-ribose) polymerase (PARP) cleavage, and PI uptake, respectively. The mechanism of UPP inhibition was analyzed by western blotting for ubiquitin, in vitro and cellular proteasomal and deubiquitinases (DUBs) activity assays. Cellular reactive oxygen species (ROS) were measured with H_2DCFDA.
Results: Curcusone D, identified as a novel UPP inhibitor, causes cell growth inhibition and apoptosis in MM cells. Curcusone D induced the accumulation of poly-ubiquitin-conjugated proteins but could not inhibit proteasomal activity in vitro or in cells. Interestingly, the mono-ubiquitin level and the total cellular DUB activity were significantly downregulated following curcusone D treatment. Furthermore, curcusone D could induce ROS, which were closely correlated with DUB inhibition that could be nearly completely reversed by NAC. Finally, curcusone D and the proteasomal inhibitor bortezomib showed a strong synergistic effect against MM cells.
Conclusions: Curcusone D is novel UPP inhibitor that acts via the ROS-induced inhibition of DUBs to produce strong growth inhibition and apoptosis of MM cells and synergize with bortezomib.
General significance: The anti-MM molecular mechanism study of curcusone D will promote combination therapies with different UPP inhibitors against MM and further support the concept of oxidative stress regulating the activity of DUBs.

1. Introduction

The ubiquitin–proteasome pathway (UPP) plays a vital role in the degradation of proteins involved in several pathways, including cellular proliferation and apoptosis. The proteasome is a validated target for multiple myeloma (MM) treatment; proteasomal inhibitors form a cornerstone of anti-myeloma therapy [1]. These inhibitors include bortezomib (PS-341), the first anti-MM proteasomal inhibitor that was FDA-approved, in 2003. Carfilzomib (Kyprolis), an epoxyketone with specific chymotrypsin-like activity, acts as an irreversible proteasomal inhibitor and was approved by the FDA in 2012 due to the improved response observed in relapsed and refractory MM patients previously treated with bortezomib [2]. However, in spite of its improved efficacy compared to alternative therapies, approximately 60% of patients do not respond to bortezomib due to the emergence of resistance.

In addition to proteasome, the UPP also includes ubiquitin, ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), ubiquitin ligases (E3s) and deubiquitinases (DUBs), which collectively work in three discrete and successive steps: 1) the substrate is tagged by the covalent attachment of multiple ubiquitin molecules via E1, E2, and E3, 2) the tagged protein is degraded by the 26S proteasomal complex, and 3) poly-ubiquitins are recycled by DUBs to free ubiquitin for reuse [3]. Multiple myeloma is the most sensitive and the most responsive disease to proteasomal inhibitors, which implies that the UPP is critical for multiple myeloma pathophysiology. E1 [4], E2s such as CDC34 [5], E3s such as Mdm-2 [6] and SCF [7], and DUBs such as USP9X [8] are overexpressed and involved in multiple myeloma pathology. Furthermore, targeting such UPP components could sensitize MM cells susceptible to bortezomib-induced cell death [4,7]. Thus, targeting the entire UPP as opposed to proteasome alone is a promising strategy for multiple myeloma treatment.

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It has been shown that the functional analysis of the UPP can be accomplished by following the steady-state levels of GFP reporter substrates, which are typically based on an intrinsic fluorescent protein with a constitutively active degradation signal that targets the proteins for ubiquitination and proteasomal degradation [9]. Such a reporter system could be used to find new UPP inhibitors with distinct mechanisms and novel structures [10].

Here, the natural compound curcusone D, a diterpene isolated from *Jatropha curcas* (Barbados nut), an herbal plant that has been used in traditional folk medicine in many tropical countries, was identified to be a novel UPP inhibitor with the Ub-G76V-YFP reporter assay. The purpose of the present study was to investigate the mechanism of UPP inhibition by curcusone D and to further characterize its anti-MM effects. Curcusone D could not inhibit the proteasome's chymotrypsin-like, trypsin-like or caspase-like activities in vitro or at the cellular level, but it did inhibit the activity of DUBs in cells. Further mechanistic studies showed that curcusone D could induce ROS, which are responsible for the inhibition of DUBs and the UPP to induce cellular growth inhibition and apoptosis. Curcusone D could inhibit multiple myeloma cell growth and induce cellular apoptosis. The combination of curcusone D with bortezomib had significant synergistic effects on MM cell growth inhibition, apoptosis and UPP inhibition.

2. Materials and methods

2.1. Reagents and cell cultures

Ubiquitin Rhodamine-110 (Ub-R110) was purchased from Boston Biochem (Cambridge, MA, USA). 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). The 2′,7′-dichlorofluoresceindiacetate (H_2DCFDA) fluorescent probe was obtained from Invitrogen (Grand Island, NY, USA). N-acetylcysteine (NAC) and trolox were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bortezomib was generously provided from the East China Biotech Company (Shanghai, China). Curcusone D was kindly provided by Dr. Mingkui Wang (Chengdu Institute of Biology, Chinese Academy of Sciences, China) and dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution. The DMSO concentration was maintained between 0.1% and 1% in all cell cultures, which caused little detectable effect on cell growth or death.

Human multiple myeloma (MM) cell lines NCI-H929, RPMI 8226, SKO, KM-3, and LP-1 were gifted from Professor Jian Hou (Chang Zheng Hospital, Shanghai, China) and were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Invitrogen, Grand Island, NY, USA) at 37 °C in a 5% CO_2 humidified atmosphere. To inhibit intracellular ROS generation, cells were pre-incubated with 10 mM NAC for 30 min prior to curcusone D treatment.

2.2. H1299-Ub-G76V-YFP, H1299-CD3δ-YFP, H1299-Ub-R-YFP, and H1299-YFP-CL1 cells

H1299 cells were maintained in RPMI-1640 medium, transfected with Ub-R-YFP, Ub-G76V-YFP, YFP-CL1, or CD3δ-YFP plasmids [11] (Addgene plasmids 11948, 11949, 11950, 11951) using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA), and selected with 800 μg/ml G418 (AMRESCO, Solon, OH, USA).

2.3. MTS assay

In a volume of 100 μL, human myeloma cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After treatment with curcusone D for the indicated time, cells were incubated with MTS at a final concentration of 0.5 mg/ml for 2 to 4 h. Optical density was determined at 490 nm (background subtraction at 690 nm) with a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The growth inhibitory ratio was calculated as follows: growth inhibitory ratio $= (A_{control} - A_{sample})/A_{control}$. IC_{50} values were derived from a nonlinear regression model (curvefit) based on a sigmoidal dose response curve (variable slope) and computed using GraphPad Prism version 5.02, GraphPad Software. The drug combination index (CI) values were calculated with the software Calcusyn (version 2) (BioSoft, Milltown, NJ, USA) according to the manufacturer's instructions.

2.4. Antibodies and immunoblotting analysis

Poly ADP-ribose polymerase (PARP), ubiquitin, and GAPDH antibodies were purchased from Cell Signaling Technology (Boston, MA, USA); the β-actin antibody was obtained from Sigma, and the HA antibody was from Abmart (Shanghai, China). All antibodies were used as recommended by the manufacturers.

Whole-cell extracts were prepared in 1X sodium dodecyl sulfate (SDS) loading buffer, subjected to electrophoresis through 12% SDS-PAGE gels, and blotted with the indicated antibodies. The quantification of blot intensity was performed using *SmartView* software of the FR-980A Gel Image Analysis System (Shanghai Furi Science and Technology Co., Ltd., Shanghai, China). PARP cleavage is reported as the ratio of cleaved PARP to total PARP and expressed as a percentage. Ubiquitin is quantified as the ratio of the drug treatment group to the DMSO control group. HA Western blot densitometry graphics have been divided into 2 parts: the first includes downregulated proteins, which are mostly USPs, the other includes insignificantly changed proteins, which are UCHs as shown in Fig. 4.

2.5. Analysis of apoptosis using FCM of AV/PI dual staining

In this study, Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) staining for FCM was used to detect apoptosis quantitatively and qualitatively. Cells were processed with an Annexin V-FITC kit (Keygene, Nanjing, China) following treatment according to the manufacturer's instructions. Next, the samples were analyzed using the FACScan flow cytometer (Becton Dickinson, Sparks, MD, USA) to quantify the apoptotic rate. Different subpopulations were distinguished using the following criteria: Q1, Annexin V-negative, but PI-positive (i.e., necrotic cells); Q2, Annexin V/PI-double positive (i.e., late apoptotic cells); Q3, Annexin V/PI-double negative (i.e., live cells); Q4, Annexin V-positive, but PI-negative (i.e., early apoptotic cells). The apoptotic rate was determined as the percentage of Q2 + Q4.

2.6. PI uptake assay

Cells were washed twice with PBS. After centrifuging at 1000 ×*g* for 5 min at 4 °C, the supernatants were collected and resuspended in 500 μl of PBS containing 10 μg/ml propidium iodide (PI) and 40 μg/ml RNase. Next, the samples were analyzed using the FACScan flow cytometer to quantify the cell death rate.

2.7. Measurement of intracellular ROS generation

After treatment with curcusone D, cells were harvested and washed with phosphate-buffered saline (PBS) and then resuspended in 1 ml of PBS. Cells were incubated with 10 μM H_2DCFDA in the dark at 37 °C for 15 min. Cells were then washed and collected in PBS and measured by FACS flow cytometry (Becton Dickinson, Sparks, MD, USA) with 485 nm excitation and 525 nm emission.

2.8. Purified proteasomal activity assay

A quantity of 1 μl of test compound was added to 10 μl purified human proteasomes (60 μg/ml for VR3, 25 μg/ml for LE3 and 5 μg/ml