ECRONICON OPEN ACCESS

EC PHARMACOLOGY AND TOXICOLOGY
Research Article

Inhibitory Effects of 1-[2-Cyano-3,12-Dioxooleana-1,9-Dien-28-Oyl] Methyl Ester (CDDO-Me) and Curcumin on Multiple Myeloma Cell Growth

Peter Pham[1], Abigail Hielscher[1], Harold Komiskey[1] and Xinyu Wang[2]*

[1]Department of Basic Sciences, Philadelphia College of Osteopathic Medicine – Georgia Campus, Suwanee, Georgia, USA

[2]Department of Pharmaceutical Sciences, College of Pharmacy, Philadelphia College of Osteopathic Medicine – Georgia Campus, Suwanee, Georgia, USA

***Corresponding Author:** Xinyu Wang, Department of Pharmaceutical Sciences, Philadelphia College of Osteopathic Medicine-Georgia Campus, School of Pharmacy, Suwanee, Georgia, USA.

Received: October 07, 2017; **Published:** October 26, 2017

Abstract

Multiple myeloma (MM) is a plasma cell cancer featured by accumulation of malignant cells preferentially in the bone marrow leading to severe bone disease in humans. In order to identify potential drug candidates for MM disease treatment, we investigated the effects of two plant-derived compounds,1-[2-cyano-3,12-dioxooleana-1,9-dien-28-oyl] methyl ester (CDDO-Me) and curcumin, in proliferation of MM cells maintained in suspension cell culture and three-dimensional (3D) microenvironments *in vitro*. The effects of CDDO-Me and curcumin on cell proliferation were evaluated in MM RPMI 8226 cells in both suspension cell culture and 3D (encapsulated) gelatin hydrogels. Cell viability was estimated using Presto Blue assay in suspension culture and the Live/Dead assay in 3D hydrogels. Induction of apoptosis was determined through PE Annexin V: 7-AAD two-color flow cytometric analysis for cells in suspension culture. Both CDDO-Me and curcumin dose- and time-dependently inhibited proliferation of MM cells in suspension cell culture. The inhibitory effects of both compounds were significantly attenuated when treating MM cells encapsulated in the 3D gelatin hydrogels. Apoptotic MM cells in suspension cell culture were increased following incubation with higher doses of both compounds. Our data indicate that CDDO-Me was a more potent cytotoxic agent for MM cells cultivated in suspension cell culture than curcumin. The gelatin-based 3D hydrogels hindered the therapeutic potency of both drugs, suggesting that MM cells may be more resistant to the cytotoxic effects of these agents when cultured in a biologically relevant 3D environment.

Keywords: *Multiple Myeloma; CDDO-Me; Curcumin; Apoptosis; 3D Hydrogel Microenvironment*

Background

Multiple myeloma (MM) is an abnormal proliferation and metastasis of the white blood cells, plasma cells in particular, within bone marrow, which may spread to the blood or urine [1]. MM is a relatively rare cancer, representing approximately 1% of all malignant tumors and 10% of hematopoietic neoplasms [2]. Despite its scarcity, close to 50% of those diagnosed with MM in the United States do not survive 5 or more years post diagnosis [2]. This particular cancer is extremely complex as there are a variety of factors that contribute to its initiation and progression including chromosomal translocations, accumulation of mutations, and other genetic abnormalities [3]. With various factors to consider and abnormalities to address, scientists have struggled to produce long-lasting therapeutic remedies [4]. Traditional cancer treatments such as chemotherapy are not effective against MM [1]. While there remains no cure for MM, the use of naturally-derived or synthetic compounds from plants may be used to overcome drug resistance and improve patient outcomes [4]. Curcumin and CDDO-derived compounds are promising plant-derived therapeutic agents that are naturally found and synthetically derived, respectively [5,6].

Curcumin is the principal active constituent in turmeric, the underground stem of a ginger-like herbaceous perennial plant with yellow flowers. People in India have used curcumin for medical purpose for years [7]. Unlike other forms of chemotherapy, curcumin presents minimal toxicity in human studies, which has allowed scientists to use this compound innocuously [5]. One of the most important properties of curcumin is the anti-cancer activity [8]. Evidence suggests that inflammation plays a role in the initiation, promotion, and progression of cancer [9]. Curcumin is able to counteract cancer initiation through its ability to down-regulate and/or inactivate NF-κB expression, one of many deregulated pathways that contribute to cancer development and progression [10]. It also reduces oxidative stress, suppresses cell cycle progression, and minimizes chronic inflammation. As such, curcumin can modulate more than one deregulated oncogenic signaling cascade and has shown the ability to combat a variety of cancers [5,7,8].

Triterpenoids are a large family of naturally occurring structures that are synthesized by plants through the cyclization of its precursor molecule called squalene [11]. Triterpenoids have been used as therapeutic agents in Asian countries for centuries. However, many natural triterpenoids such as oleanolic acid (OA) and ursolic acid (UA) possess relatively weak anti-inflammatory and anti-cancer effects in comparison to other naturally occurring therapeutic compounds [12]. It has been discovered that some derivatives of OA and UA display more potent effects than their triterpenoid parent compounds. One of the most potent synthetically-derived triterpenoids is 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO). Studies on CDDO have demonstrated its powerful anti-inflammatory and anti-cancer effects in a wide range of cells [13]. One of the most well studied derivatives of this compound is CDDO-Methyl ester (CDDO-Me). This compound has been documented to possess anticancer activities through prevention of proliferation and induction of apoptosis in various cancer cells including ovarian and prostate cancer cells [14-16]. In addition, it was reported to inhibit pancreatic tumor growth through down-regulation of microRNA-27a [17]. Its effects on MM cell growth, however, have not been reported to our knowledge.

The present study investigated the anti-myeloma effects of curcumin and CDDO-Me in MM cells. Both compounds were examined for time and dose-dependent responses in suspension cultures of MM cells. To more accurately capture the *in vivo* environment, MM cells were encapsulated in 3D gelatin hydrogels prior to drug treatment. The anti-cancer effects of curcumin and CDDO-Me were determined by measuring the viability of MM cells following drug treatment at various concentrations and time points and through examination of cell death mechanisms. By studying the therapeutic efficacy of curcumin and CDDO-Me, a better understanding of the mechanisms that underlie MM responsiveness to these potential therapeutic compounds will be gained. In addition, the application of 3D cultures better mimics the *in-vivo* microenvironment for MM cells and enables screening of potential drug candidates in a biologically relevant environment.

Methods

Chemicals and reagents

Curcumin, porcine-derived gelatin, Calcein acetoxymethyl ester (Calcein-AM), ethidium bromide, RPMI-1640 medium and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Microbial transglutaminase was obtained from Ajinomoto (Itasca, IL, USA). CDDO-Me was obtained from Cayman Chemicals (Ann Arbor, MI, USA). Presto Blue Cell Viability Reagent, fetal bovine serium (FBS), penicillin, and streptomycin were all purchased from Thermo Fisher Scientific (Grand Island, NY, USA). PE Annexin V Apoptosis Detection Kit I was acquired from BD Biosciences (San Jose, CA, USA). Live/dead assay was obtained from Life Technologies (Carlsbad, CA, USA).

Cell line and cell culture

The human MM cell line (RPMI 8226) was obtained from American Type Culture Collection (Manassas, VA, USA). MM cells were grown in RPMI-1640 medium supplemented with 10% FBS and antibodies (100 U/ml penicillin and 100 μg/ml streptomycin). MM cells were cultured at 37ºC in a humidified atmosphere in the presence of 5% CO_2 and 95% air. The cell culture was supplemented with fresh media every 2 to 3 days to maintain appropriate cell concentration in the media according to the manufacturer's instruction (ATCC, Manassas, VA, USA).

Cytotoxicity assays in suspension cell culture

A total of 40,000 viable cells were plated in each well of 48-well plates immediately prior to treatment. MM cells were treated with either curcumin (1 μM, 5 μM, 10 μM, 20 μM) or CDDO-Me (0.1 μM, 0.5 μM, 1 μM, 5 μM). Both compounds were dissolved in DMSO to prepare stock solutions and diluted further in cell culture media immediately prior to administration to the cells. MM cells were incubated with the compounds for 24, 48, and 72-hour periods. Presto Blue Cell Viability Reagent (10%) was utilized to measure the viability of MM cells following the manufacturer's protocol. After one hour of incubation at 37ºC, the resulting fluorescent signaling was measured at 545 nm excitation and 590 nm emissions using a Synergy HT plate reader (BioTek Instruments Inc., Winooski, VM, USA). All cytotoxicity experiments were performed at least 3 times in more than three replicates.

Cell apoptosis assays

The PE Annexin V Apoptosis Kit was used to detect apoptosis of MM cells induced by CDDO-Me and curcumin in suspension culture following the manufacturer's protocol (BD Biosciences). Briefly, 1×10^6 MM cells were seeded in each well of a 6-well plate and treated with curcumin (1 μM, 5 μM, 10 μM, 20 μM) or CDDO-Me (0.1 μM, 0.5 μM, 1 μM, 5 μM), respectively. After incubation for 48 hours, cells were centrifuged, and pellets were washed twice with PBS and re-suspended in 1X Annexin V binding buffer. Next, the PE Annexin V and 7-AAD dyes were added to the cells and allowed to incubate for 15 minutes at room temperature in the dark. After a final addition of 1X binding buffer, the cells were analyzed using the BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Cell encapsulation

In order to evaluate the effects of curcumin and CDDO-Me in a 3D microenvironment, 50,000 MM cells were encapsulated in pre-warmed 7.5% gelatin polymerized with 20 μg/mL microbial transglutaminase (mTG) prior to drug treatment. A volume of 0.25 mL of the gel/cell solution was quickly pipetted to each well of 24-well plates, where the gels were allowed to polymerize for approximately 30 minutes at 37ºC. Once the gelatin polymerized, 1 mL of complete media was added to the top of each gel and the gel encapsulated cells were placed in a humidified atmosphere at 37ºC with 5% CO_2 and 95% air.

3D cytotoxicity assay

After one hour of MM encapsulation in gelatin gels, 1mL of media containing curcumin (5 μM, 10 μM, 20 μM) or CDDO-Me (0.1 μM, 0.5 μM, 1 μM) was added to each well. Following 24, 48, and 72-hour incubation periods with each compound, the Live/Dead Assay was used to determine the percentage of live cells in each treated well. The EVOS FL Auto Cell Imaging System (Life Technologies) was used to capture 3 images per well at 10X magnification in triplicate samples during one experiment. The particle count tool in Image J (NIH) was used to determine the percent of live and dead cells. The results were presented from four independent experiments.

Statistical analysis

Data are presented as the means plus SEM. Differences among control and treatment groups were analyzed using two-way analysis of variance for multiple comparisons through GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). A P value ≤ 0.05 was considered statistically significant.

Results

Curcumin and CDDO-Me inhibit growth of MM cells maintained in suspension cell culture

Data from cytotoxicity assays performed on MM cells treated with various doses of curcumin showed time and dose-dependent responses (Figure 1A). Only the highest concentration of curcumin (20 μM) produced a significant difference compared to control at 24, 48, and 72-hour time points. Specifically, cell viability decreased by 40%, 60%, and 65% after 24, 48 and 72-hour incubation with curcumin, respectively (Figure 1A). Interestingly, lower concentrations of curcumin (1 μM and 5 μM) appeared to improve cell viability (Figure 1A).

CDDO-Me also demonstrated time and dose-dependent inhibition of MM cell proliferation (Figure 1B). After 24, 48, and 72-hour incubations, cell viability significantly decreased when treated with 1 μM and 5 μM CDDO-Me (Figure 1B). Results showed that 1 μM and 5 μM CDDO-Me effectively decreased cell viability to 45% and 50% by 24 hours and 85% and 90% by 72 hours, respectively. MM cells treated with 0.1μM CDDO-Me, however, exhibited increased cell viability after 24 48, and 72 hours (Figure 1B).

Curcumin and CDDO-Me modestly reduce cell viability of MM cells maintained in a 3D gelatin hydrogel

Given the potent cytotoxic effects of curcumin and CDDO-Me on MM cells grown in traditional suspension culture, we next tested the therapeutic efficacy of these compounds on MM cells encapsulated in 3D gelatin hydrogels. Toxicity results for curcumin on MM cells encapsulated in gelatin hydrogels are shown in Figure 1C. Percent of live MM cells was reduced slightly from above 90% (control group) to the same 84% (highest concentration of curcumin tested) at both 48 and 72 hour post treatment, respectively (Figure 1C). Toxicity results for CDDO-Me on MM cells encapsulated in gelatin hydrogels are shown in figure 1D. Similarly, percent of live MM cells was reduced slightly from above 90% (control group) to 83 and 85% (highest concentration of CDDO-Me tested) at 48 and 72 hour post treatment, respectively (Figure 1D).

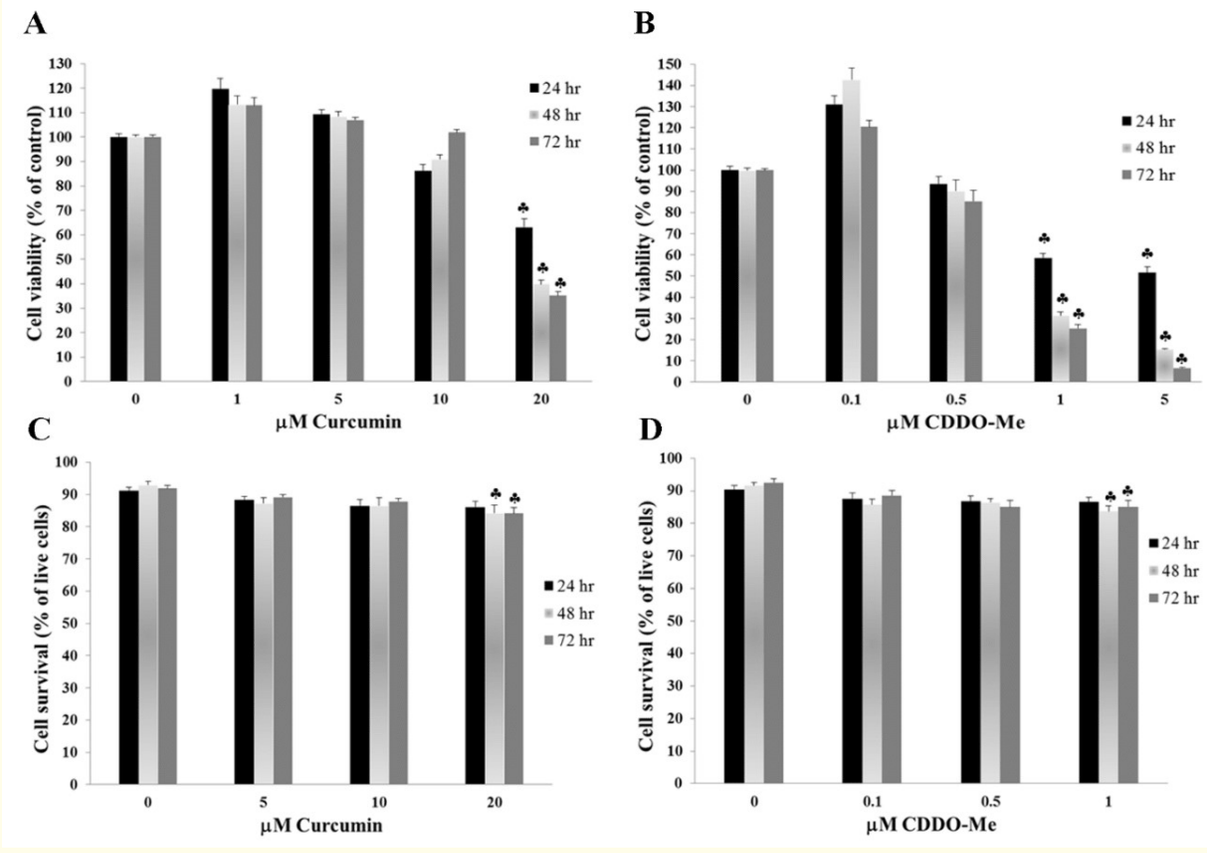

Figure 1: *Effects of curcumin and CDDO-Me on MM cell growth in suspension cell culture and 3D hydrogels. Curcumin and CDDO-Me demonstrated time and dose-dependent toxicity in suspension cultured MM cells at 24, 48, and 72 hours post-incubation. A) Curcumin at the highest concentration (20 μM) demonstrated significant toxicity against MM cells maintained in suspension cell culture at 24, 48, and 72 hours post-incubation. B) At three time points tested, higher concentrations (1 μM and 5 μM) of CDDO-Me significantly decreased viability of MM cells grown in suspension cell culture. C) Curcumin slightly decreased the percent of live MM cells encapsulated in the 3D hydrogel after 48 and 72 hours of treatment. D) Similarly, CDDO-Me only slightly decreased the percent of live MM cells encapsulated in the 3D hydrogel. ♣: p ≤ 0.05 versus vehicle control (0 μM curcumin or CDDO-Me).*

Curcumin and CDDO-Me induce apoptosis of MM cells maintained in suspension cell culture

Both curcumin and CDDO-Me induced apoptosis in MM cells in a dose-dependent manner 48 hour post treatment (Figure 2A and 2B). Based on flow cytometry analyses, lower concentrations of curcumin (1 μM, 5 μM, 10 μM) induced apoptosis in 14 - 15% of cells (early plus late apoptotic), whereas the highest concentration (20 μM) induced apoptosis in 20% of cells (Figure 2A). A greater percentage of MM cells treated with CDDO-Me underwent apoptosis compared to those treated with curcumin (Figure 2B). The lowest dosage (0.1 μM) of CDDO-Me triggered apoptosis in 16% of cells, whereas 71% of cells were in either the early or late stages of apoptosis when treated with the highest dosage (5 μM) of CDDO-Me (Figure 2B). CDDO-Me at 0.5 μM induced a higher percentage of apoptotic MM cells than curcumin at 20 μM (29% vs 20%), indicating that CDDO-Me is a more potent inducer of MM cell apoptosis than curcumin.

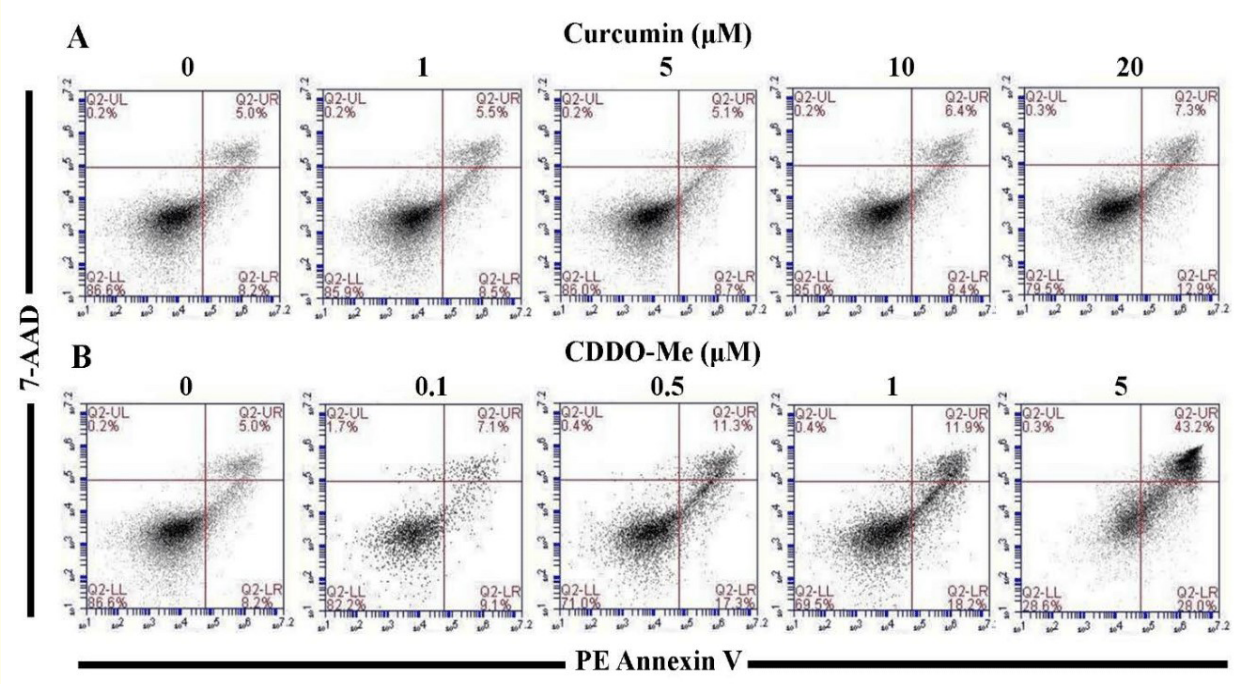

Figure 2: *Curcumin and CDDO-Me induce apoptosis of MM cells. A) Curcumin dose-dependently triggered apoptosis in MM cells maintained in suspension cell culture. Lower doses of curcumin (1 μM, 5 μM, 10 μM) induced apoptosis in 14 - 15% of cells, whereas the highest concentration (20 μM) induced apoptosis in 20 % of cells. B) CDDO-Me triggered apoptosis in MM cells in a dose-dependent manner. CDDO-Me initiated apoptosis in a higher number of cells compared to curcumin. The lowest dosage (0.1 μM) triggered cell death in 16% of cells, whereas 71% of cells were in either the early or late stages of apoptosis when treated with the highest dosage (5 μM) of CDDO-Me.*

Discussion

Curcumin and CDDO-Me demonstrated significant cytotoxic effects in MM cells in suspension cell culture environments. Through flow cytometry analyses of MM cells treated with both compounds, cell death was found to be partly attributed to apoptosis. Although both compounds were able to slightly reduce the percent of live cells in 3D microenvironments at high dose with long exposure, they were

much more potent in the traditional suspension cell culture. In this environment, the non-adherent MM cells move freely in the media. As the compounds are administered to each well, MM cells come into direct contact with them, allowing the cytotoxic agents to deliver their therapeutic effects without any physical hindrances. In our cytotoxicity assays, we found that the highest doses of both drugs significantly decreased viability of MM cells. Interestingly, the lowest doses of both drugs appeared to improve the viability of MM cells. The stimulation of human tumor cell proliferation at low doses of anti-tumor agents has been observed in various tumor cell lines [18]. Our observation of low-dose stimulation and high-dose inhibition of MM cell proliferation for both compounds indicates a hermetic dose-response relationship. This biphasic concentration response relationship has also been reported in many other tumor cell lines [18]. Although a number of possible explanations are proposed, there is no universal mechanism that accounts for this phenomenon. We speculate that lower concentrations of curcumin and CDDO-Me likely disrupt cellular homeostasis as stress stimuli and trigger a modest overcompensation response of the host. On the contrary, high doses of these compounds overcome MM self-protective mechanisms leading to inhibition of cell proliferation [18].

It remains unclear whether these compounds are innocuous to normal cells *in vivo*. From cytoprotective assays involving drug treatment against oxidative-stressed astrocytes, 5 μM CDDO-Me unexpectedly reduced cell viability of live, healthy astrocytes (unpublished data). This same concentration also significantly decreased cell viability of MM cells to less than 10% in suspension cell culture after 72 hours. This evidence suggests that CDDO-Me, at this particular concentration or higher, may be toxic to not only cancer cells, but also to healthy cells. Curcumin on the other hand, even at high doses (20 μM), did not present any harmful effects against astrocytes (unpublished data). Although CDDO-Me appears to be a more potent anti-cancer drug, its therapeutic efficacy may be limited due to its toxic effects against healthy cells.

Although traditional cell culture is useful for cytotoxicity purposes, these remain highly limited because of their poor representation of the *in vivo* environment [19]. The gelatin based 3D hydrogel was used to more accurately capture the *in viv*o environment of the cancer cells. Utilizing this technique bridges the gap between traditional cell culture and animal models. The gel serves to imitate the extracellular matrix surrounding all cells [19]. When MM cells were treated with curcumin and CDDO-Me in the 3D hydrogel, the toxic effects of the compounds were much less potent compared to the same treatment in the suspension cell culture. The gel may have become a barrier that prevented the drugs from fully penetrating into the cells to deliver their therapeutic effects. Previous studies have implicated that the microenvironment contributes significantly to MM tumor survival and progression [2]. Recently, a new 3D model of hyaluronic acid -based hydrogels was developed to study MM cell responses to drug treatment [20]. Although our model mimics some of the features of the tumor microenvironment, additional work is warranted to more accurately capture the mechanical and biochemical attributes of the MM tumor environment.

Conclusion

Curcumin and CDDO-Me demonstrated dose and time-dependent toxicity in MM cells grown in suspension cell culture, and CDDO-Me was found more potent than curcumin. The gelatin-based 3D microenvironment, however, hindered the therapeutic potency of both compounds. Both curcumin and CDDO-Me have proven to be effective against MM cell growth. However, the ability of these compounds to target MM *in vivo* remains unknown. Further studies will investigate special delivery methods in order to maximize the anticancer potency of curcumin and CDDO-Me. Plant-derived drugs remain very encouraging for treating certain cancers. In this study, curcumin and CDDO-Me showed potential in their ability to induce apoptosis in MM cells grown in suspension cell culture. However, there is more to investigate on mechanisms involved in their therapeutic effects and the application of gelatin based 3D hydrogels as a model to mimic the *in vivo* microenvironment. If these mechanisms can be better understood and the 3D model can be fully developed, more effective therapeutic drugs can enter the market to treat those suffering from MM.

Competing Interest

The authors have no conflict of interest.

Acknowledgments

We thank Yan Wu for technical support on flow cytometric analysis. This project is supported by funding from Center for Chronic Disorders of Aging at Philadelphia College of Osteopathic Medicine.

Bibliography

1. Palumbo A and K Anderson. "Multiple Myeloma". *New England Journal of Medicine* 364.11 (2011): 1046-1060.
2. Pulte D., *et al*. "Trends in Survival of Multiple Myeloma Patients in Germany and the United States in the First Decade of the 21st Century". *British Journal of Haematology* 171.2 (2015): 189-196.
3. Podar K., *et al*. "Bone Marrow Microenvironment and the Identification of New Targets for Myeloma Therapy". *Leukemia* 23.1 (2009): 10-24.
4. Hideshima T., *et al*. "Understanding Multiple Myeloma Pathogenesis in the Bone Marrow to Identify New Therapeutic Targets". *Nature Reviews Cancer* 7.8 (2007): 585-598.
5. Goel A and BB Aggarwal. "Curcumin, the Golden Spice from Indian Saffron, Is a Chemosensitizer and Radiosensitizer for Tumors and Chemoprotector and Radioprotector for Normal Organs". *Nutrition and Cancer* 62.7 (2010): 919-930.
6. Wang YY., *et al*. "Preclinical Evidences toward the Use of Triterpenoid Cddo-Me for Solid Cancer Prevention and Treatment". *Molecular Cancer* 13 (2014): 30.
7. Ghosh S., *et al*. "The Beneficial Role of Curcumin on Inflammation, Diabetes and Neurodegenerative Disease: A Recent Update". *Food and Chemical Toxicology* 83 (2015): 111-124.
8. Heger M., *et al*. "The Molecular Basis for the Pharmacokinetics and Pharmacodynamics of Curcumin and Its Metabolites in Relation to Cancer". *Pharmacological Reviews* 66.1 (2014): 222-307.
9. Coussens LM and Z Werb. "Inflammation and Cancer". *Nature* 420.6917 (2002): 860-867.
10. Bharti AC and BB Aggarwal. "Nuclear Factor-Kappa B and Cancer: Its Role in Prevention and Therapy". *Biochemical Pharmacology* 64.5-6 (2002): 883-888.
11. Wang Y., *et al*. "A Synthetic Triterpenoid, 2-Cyano-3,12-Dioxooleana-1,9-Dien-28-Oic Acid (Cddo), Is a Ligand for the Peroxisome Proliferator-Activated Receptor Gamma". *Molecular Endocrinology* 14.10 (2000): 1550-1556.
12. Nishino H., *et al*. "Inhibition of the Tumor-Promoting Action of 12-O-Tetradecanoylphorbol-13-Acetate by Some Oleanane-Type Triterpenoid Compounds". *Cancer Research* 48.18 (1988): 5210-5215.
13. Suh N., *et al*. "A Novel Synthetic Oleanane Triterpenoid, 2-Cyano-3,12-Dioxoolean-1,9-Dien-28-Oic Acid, with Potent Differentiating, Antiproliferative, and Anti-Inflammatory Activity". *Cancer Research* 59.2 (1999): 336-341.
14. Gao X., *et al*. "Ros Mediate Proapoptotic and Antisurvival Activity of Oleanane Triterpenoid Cddo-Me in Ovarian Cancer Cells". *Anticancer Research* 33.1 (2013): 215-221.
15. Deeb D., *et al*. "Oleanane Triterpenoid Cddo-Me Inhibits Growth and Induces Apoptosis in Prostate Cancer Cells through a Ros-Dependent Mechanism". *Biochemical Pharmacology* 79.3 (2010): 350-360.

16. Zou W., *et al*. "C-Jun Nh2-Terminal Kinase-Mediated up-Regulation of Death Receptor 5 Contributes to Induction of Apoptosis by the Novel Synthetic Triterpenoid Methyl-2-Cyano-3,12-Dioxooleana-1, 9-Dien-28-Oate in Human Lung Cancer Cells". *Cancer Research* 64.20 (2004): 7570-7578.

17. Jutooru I., *et al*. "Methyl 2-Cyano-3,12-Dioxooleana-1,9-Dien-28-Oate Decreases Specificity Protein Transcription Factors and Inhibits Pancreatic Tumor Growth: Role of Microrna-27a". *Molecular Pharmacology* 78.2 (2010): 226-236.

18. Calabrese EJ. "Cancer Biology and Hormesis: Human Tumor Cell Lines Commonly Display Hormetic (Biphasic) Dose Responses". *Critical Reviews in Toxicology* 35.6 (2005): 463-582.

19. Yamada KM and E Cukierman. "Modeling Tissue Morphogenesis and Cancer in 3d". *Cell* 130.4 (2007): 601-610.

20. Narayanan NK., *et al*. "Characterization of Multiple Myeloma Clonal Cell Expansion and Stromal Wnt/Beta-Catenin Signaling in Hyaluronic Acid-Based 3d Hydrogel". *In Vivo* 28.1 (2014): 67-73.

Volume 4 Issue 6 October 2017