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Original article

# Bardoxolone methyl induces neuritogenesis in Neuro2a cells

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ABSTRACT

*Background:* Bardoxolone methyl (RTA 402, CDDOMe) has been long known for its anti-inflammatory and exceptional cytotoxic activity. The biological responses to CDDOMe are truly dose dependent. And owing to the structural modifications introduced in its parent molecule oleanolic acid, CDDOMe is able to form reversible adducts with cellular proteins containing redox sensitive cysteine residues. This nature of CDDOMe makes it a multifunctional molecule targeting multiple signaling pathways. This study was initiated to study the response of Neuro2a, a mouse neuroblastoma cell line to CDDOMe.

*Methods:* Neuro2a cells were treated with CDDOMe and all trans retinoic acid (ATRA) for 4 days and observed for neurite outgrowth. The neurite length was estimated using ImageJ software (Neuron growth plugin). Cell viability was investigated using MTT dye reduction and trypan blue dye exclusion method. Gene expression of differentiation markers was analyzed using quantitative PCR. Cellular localization of Tuj1 and synaptophysin in differentiated Neuro2a cells was observed using immunofluorescence.

*Results:* CDDOMe ceased proliferation and induced dramatic neurite outgrowth in Neuro2a cells. These morphological changes were accompanied by time dependent increase in the mRNA levels of tyrosine hydroxylase, neurofilament 200 and synaptophysin. Besides, cytoskeleton protein Tuj1 and the synaptic vesicle protein synaptophysin were also observed to be localized in the neurites induced by CDDOMe.

*Conclusions:* These early shreds of evidence suggest that CDDOMe induces differentiation in Neuro2a cells at concentrations ranging from 0.2 to 0.4 μM and indeed contributes the existing knowledge on CDDOMe induced activities in cells.



## Introduction

CDDOMe is a C28 methyl ester of CDDO which is a synthetic derivative of a naturally occurring oleanane triterpenoid [1]. CDDOMe exhibits dual roles with lower concentrations being anti-inflammatory whereas higher concentrations are found to be extremely cytotoxic in various cancer cell lines. The preventive and curative efficacy of CDDOMe has been evaluated in multiple cancers using carcinogen induced tumor or transgenic or xenograft models [2,3]. CDDOMe inactivates the negative regulator of Nrf2 i.e. Keap1 *via* covalent cysteine modification thereby increasing the expression of Nrf2 targeted genes which majorly includes detoxifying enzymes fighting for cell survival. Besides, CDDOMe blocks NFκB activation by interacting with cysteine in the IKKβ activation loop thereby preventing the IκBα phosphorylation. Hence, CDDOMe is referred as an antioxidant inflammation modulator [4]. Further, CDDOMe inhibits pro-survival pathways and induces apoptosis in *in vitro* and *in vivo* conditions by targeting multiple signaling pathways [5]. Thence the effect of CDDOMe has been researched on inflammatory disorders, neurodegenerative diseases, cancers, diabetes and many other diseases. Structure activity studies have shown that α, β-unsaturated carbonyl groups on enone rings A and C form reversible adducts with cellular nucleophilic targets through Michael addition. CDDOMe can modify proteins by reacting with the redox sensitive sulfhydryl groups of cysteine residues on proteins rendering them nonfunctional. This nature of CDDOMe defines the pharmacological importance of CDDOMe, as it helps it

*Abbreviations:* Akt, v-Akt Murine Thymoma Viral Oncogene; ARE, Antioxidant Responsive Element; BDNF, Brain Derived Neurotrophic Factor; CDDO, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; CHOP, C/EBP homologous protein; DR5, Death Receptor 5; ERK, Extracellular signal Regulated Kinases; IKKβ, IκB kinase β; JNK, cJun N terminal Kinas; Keap, Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1; MAPK, Mitogen Activated Protein Kinase; mTOR, Mechanistic target of Rapamycin; NFκB, Nuclear Factor Kappa B; Nrf2, Nuclear factor erythroid 2-Related Factor 2; ROS, Reactive oxygen species; STAT, Signal Transducer and Activator of Transcription 3.

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to not only interact with several regulatory proteins but also absorb ROS [6].

The Keap1-Nrf2-ARE pathway activation is a cyto-protective defense response against electrophilic stresses that damage cellular proteins and enzymes. Therefore, CDDOMe as a Nrf2 activator is been tested against diseases involving oxidative stress and inflammation [7]. Preliminary studies with ischemia–reperfusion injury (IRI) mice model suggest that CDDOMe treatment decreased infarct volume and improved neurological symptoms due to early activation of Nrf2 and its target genes in neurons and astrocytes [8]. Extended study demonstrated that CDDOMe reduced intracranial hemorrhage volume caused by cerebral ischemia/reperfusion injury and also protected the cellular components of blood brain barrier [9]. A biotransformed form of CDDOMe i.e. RS9 was found to be more potent Nrf2 activator and less cytotoxic compared to CDDOMe and exhibited neuroprotective effect by inhibiting both oxidative stress and neuroinflammation in a cerebral ischemia reperfusion model [10]. Additionally, a recent report testifies the potential of CDDOMe in preventing high fat diet induced cognitive impairment in mice model. CDDOMe improved neuronal plasticity by boosting downstream BDNF signal transduction, increasing activated AMPK, and reducing inflammation in the hippocampus and prefrontal cortex region [11]. With respect to differentiation, this multifunctional molecule hasn't been explored except for one study that reports it as granulomonocytic differentiation inducer [12]. Limited studies report that CDDOMe induces growth arrest and apoptosis in several neuroblastoma cell lines [13,14]. In the present study, we have investigated the effect of CDDOMe on Neuro2a which is a neural crest derived cell line from mouse neuroblastoma.

## Materials and methods

### *Cell culture and reagents*

Neuro2a cell line was purchased from NCCS (Pune) and was maintained in Dulbecco's Modified Eagle Medium (DMEM, HiMedia) at 37 °C in 5% humidified $CO_2$ atmosphere. Media was supplemented with 10% fetal bovine serum (FBS, HiMedia), 1× antibiotic-antimycotic (HiMedia) and 1× glutamax (Gibco). CDDOMe was purchased from Cayman. ATRA was purchased from Sigma Aldrich. Stocks were prepared in DMSO.

### *Treatment and neurite staining*

Neuro2a cells were seeded in six well plates at a density of $1 \times 10^5$ cells per well and treated with different concentrations of CDDOMe and ATRA and observed for neurite outgrowth. To observe cell morphology and neurites; cells were washed, fixed with 50% ethanol and stained with methylene blue solution. 5 random fields for each condition were imaged on EVOS FLoid Imaging station equipped with monochrome CCD camera (Thermo Fisher Scientific). Quantitative analysis was performed by tracing neurites using ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA) with neuron growth plug-in (Universidad Nacionalg Autónoma de México, UNAM). Controls received the DMSO alone.

### *Cell viability*

Cell viability was quantified by MTT assay that depends on the reduction of tetrazolium dye – MTT (3-[4,5-dimethyl thiazole-2-yl]-2,5-diphenyl tetrazolium bromide) in viable cells by mitochondrial NADPH dependent cellular oxido-reductase enzymes which is indicative of the metabolic activity [15]. Briefly, $1 \times 10^3$ cells were seeded in 96 well plates and treated with CDDOMe and ATRA at varying concentrations for indicated time periods. Experiment was terminated by adding MTT reagent (5 mg/ml) and incubating the plate for further 3 h at 37 °C. The medium in each well was replaced with 200 μl of DMSO to facilitate cell lysis and to solubilize purple colored formazan dye. The plate was read at 570 nm in Elisa Plate Reader (Biorad). Controls received the DMSO alone.

Absolute number of viable cells was counted using trypan blue exclusion method. Briefly, $1 \times 10^4$ cells were seeded in 24 well plates and treated with CDDOMe (0.4 μM) and ATRA (25 μM) for 4 days. At different experimental time points both adhered and suspended cells were collected and stained with 0.4% trypan blue solution. Viable cells were counted manually using hemocytometer.

### *RNA extraction, reverse transcriptase PCR and gene expression analysis*

After inducing differentiation cells were harvested at different time points. Total RNA from (both treated and control wells) cultured cells were isolated using RNA isoplus (Takara). RNA quality and quantity were assessed by Nanodrop UV–vis spectrophotometer (Thermo Fisher Scientific). Prime Script RT reagent kit (Takara) was used to reverse transcribe 2 μg of total RNA from each sample into cDNA. Quantitative PCR was performed using SYBR Premix Ex Taq (Tli RNaseH Plus, Takara) on Applied Biosystems Step one plus PCR machine. The GAPDH gene was amplified as an internal standard reference gene (invariant control). Fold changes in the target gene expression were normalized to GAPDH gene expression using comparative CT method ($2^{-D\Delta CT}$ method) [16].

### *Immunofluorescence*

Neuro2a cells were seeded on gelatin coated coverslips in complete media and following day were treated with CDDOMe and ATRA. On day 4, cells were washed with DPBS and fixed with 4% formaldehyde. Primary antibodies employed for immunofluorescence are: anti β3-Tubulin rabbit monoclonal antibody (Tuj1, 5568S; Cell Signaling Technology, Danvers, MA, USA) and anti Synaptophysin antibody (NBP2-25170; Novus Biologicals, Littleton, CO, USA). An Alexa Fluor 594 Conjugate goat anti-rabbit (8889S, Cell Signaling Technology, Danvers, MA, USA) was used as secondary antibody. Every step was followed according to manufacturer's instructions. The coverslips were mounted in fluoroshield (Sigma Aldrich, St Louis, MO, USA). Fluorescent images were acquired on EVOS FLoid Imaging Station.

### *Statistical analysis*

Statistical tests were performed using Graph Pad Prism 6 software. One-way ANOVA followed by Dunnet's or Tukey's multiple comparison test were applied to compare more than two groups. Two-way ANOVA followed by Tukey's *post-hoc* test was applied to compare multiple groups (two parameters). A value of $p < 0.05$ was considered statistically significant. Results are expressed by mean ± SEM.

## Results

### *CDDOMe inhibits proliferation and decreases viability of Neuro2a cells*

To investigate the effect of CDDOMe on cell viability; initially, MTT assay was performed. At first, we treated Neuro2a cells for 72 h with CDDOMe at a broad concentration range. We observed a dose dependent toxicity where higher concentrations of 0.75 μM–2 μM were extremely cytotoxic and lower concentrations inhibited