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Evaluation of the Cytotoxicity of Levodopa and its Complex with Hydroxypropyl-ß-Cyclodextrin (HP-ß-CD) to an Astrocyte Cell Line

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Abstract

A simple, reliable a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, (MTS) assay was conducted to evaluate the potential cytotoxic effects of levodopa, a "gold standard therapy" for Parkinsonism, and its complex with Hydroxypropyl-β-Cyclodextrin (HP-β-CD) on an astrocyte cell line. The cells were incubated in a range of concentrations from 4.69 to 300 µg/mL levodopa, HP-β-CD or the complex for up to 72 hours. At every 24-hour interval, the optical density (OD), which reflects the number of viable cells, was recorded. In general, linear dose-dependent cytotoxicity profiles were observed for the cells subjected to levodopa or the complex, whereas a slightly triphasic response was observed for the cells exposed to HP-β-CD. A significant difference ($P < 0.05$) in cytotoxicity was detected between the HP-β-CD-treated group and the levodopa-treated group. In particular, we observed that the cells treated with the complex, even at the highest concentrations (> 200 µg/mL), exhibited improved tolerability in a time-dependent manner, which may indicate the potential ability of HP-β-CD to mask the toxic effects of levodopa via complexation.

Keywords: *cyclodextrin, levodopa, astrocytes, cytotoxicity*

Introduction

In the decades since it was first introduced to the clinical world, levodopa (LD) remains the most effective treatment for symptomatic relief of Parkinson's disease (PD). LD, or 3,4-dihydroxy-L-phenylalanine, is a naturally-occurring amino acid that serves as an optimal dopamine precursor to help manage (or even prevent) the motor complications of PD patients. However, reports have shown that within 5-10 years, most PD patients begin to experience motor fluctuations, including predictable wearing "off" phenomena, an unpredictable "off" period, a delayed time to "on", and response failures or "no-on" phenomena (1–5). Although these irregularities have been considered by investigators as a multifactorial pathogenesis, the development of these irregularities are believed to strongly depend on the pharmacokinetic properties of LD (6,7).

When orally administered to humans, LD is absorbed via a facilitated, saturable uptake process localised to the proximal upper gastrointestinal tract. Its extensive pre-systemic metabolism in the proximal small intestine, its very short half-life due to rapid metabolism, and its poor solubility enable only a small fraction of LD to ultimately reach the brain because most of it is absorbed by skeletal muscle, the liver and the kidneys (8,9). Efforts to improve upon LD therapy have emphasised the need to reformulate LD into new preparations that display controlled release or enhanced solubility. A controlled-release form of LD may have a longer half-life and may provide

more stable plasma levels; alternatively, an LD compound with enhanced solubility may enable faster and more reliable absorption, which would be beneficial for patients exhibiting "no-on" or "delayed-on" phenomena.

For years, cyclodextrins (CDs) have been extensively studied for their unique ability to form complexes with a wide range of organic, inorganic and biologic guest molecules. CDs are crystalline, homogenous and non-hygroscopic substances in nature. These cyclic oligosaccharides possess a relatively hydrophilic outer layer and a hydrophobic inner cavity (10). Complexations with CDs are achieved via a host-guest reaction in which the guest molecules are temporarily caged either in whole or in part. These stable non-covalent complexes display improved physicochemical characteristics compared to the unmanipulated drugs, potentially including increased solubility for poorly soluble drugs, stability in solution, and controlled release of the drug (11,12).

Previously in our laboratory, the successful formation of a hydroxypropyl-beta-cyclodextrin (HP-β-CD)/levodopa complex was confirmed via several analytical chemistry analyses (data not shown), which revealed that the bioavailability of LD was altered upon complexation. In this study, we further extended our work to evaluate the potential cytotoxic effect of this newly formulated LD to an astrocyte cell line using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

Materials and Methods

Materials

LD was purchased from the United States Pharmacopoeia (Rockville, MD, USA). Phosphate-buffered saline (PBS) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich, St Louis, MO, USA. The normal rat astrocyte cell line originated from the American Type Culture Collection (ATCC, Cat. no. CRL 2600) (USA). Eagle's Minimal Essential Medium (EMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS) was purchased from Mediatech, USA. Penicillin/streptomycin and trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05 % trypsin and 0.53 mM EDTA-4Na) were purchased from Gibco-BRL, USA. MTS was obtained from Promega, USA.

Cell culture

The cells were grown as adherent cultures in sterile 25 cm2 culture flasks (Greiner Bio-One, Austria) using growth medium consisting of EMEM supplemented with 10% heat-inactivated FCS and 100U/mL penicillin/streptomycin. The cultures were maintained in a humidified atmosphere containing 5% CO2 at a controlled temperature of 37 + 0.10C. Once the cells reached confluence, the culture medium was removed, and the adherent cells were detached. The cells were removed using a standard method via the addition of trypsin: EDTA. Then, this mixture was incubated for approximately 10 min at 37 °C. After centrifugation at 1000 rpm for 8 min at 4 °C, the pellet was used for cell counting. Then, the dissociated cells were seeded at a density of 5 × 104 viable cells/cm2 in the medium. The medium was changed 24 hours after seeding.

Cytotoxicity assay

The potential cytotoxic effects were examined via an MTS assay. This colorimetric assay is based on the conversion of a tetrazolium salt to a coloured, aqueous soluble formazan product formation, which reflects the number of viable cells (13). In brief, LD, HP-β-CD or the complex was added to the cells at seven different concentrations ranging from 4.69 to 300 µg/mL. The cells were incubated for up to 72 hours. At every 24 hours interval, the optical density (OD) was recorded using an ELISA plate reader to determine the percentage of cell viability. All compounds used were freshly prepared prior to the experiment and were covered with aluminium foil for protection from light. A non-treated group was used as a control.

Statistical analysis

One-way ANOVA followed by Tukey's test (GraphPad prism 5) was performed to identify statistical significance. A P value < 0.05 was considered to be significant.

Results

Figure 1, 2, and 3 illustrate the percentage of cell viability relative to control treatment after 24, 48, and 72 hours of incubation in LD, HP-β-CD, or the complex a concentrations ranging from 4.69 to 300 µg/mL. Generally, the responses observed were dose- and time- dependent, in which the cytotoxicity profiles appeared to be strongly associated with the type of treatment applied. As shown in Figure 1, the cells treated with LD or the complex displayed a linearly dose-dependent cytotoxicity profile. Alternatively, the cells treated with HP-β-CD displayed a slightly triphasic

response. In particular, a cytotoxic effect of LD and the complex applied at high concentrations (> 200 µg/mL) on the cells was clearly observed, as exhibited by the apparent decrease in the percentage of cell viability. A similar pattern was found after incubation for 48 or 72 hours between the LD- and HP-β-CD-treated groups (Figure 2 and 3). However, in the complex-treated group, we observed an improved tolerability of these cells to the compound, even at high concentrations (> 200 µg/mL), as demonstrated by an increase in the percentage of cell viability compared to 24 hours. A significant difference ($P < 0.05$) in cytotoxicity was detected between the HP-β-CD-treated group and the LD-treated group at 48 and 72 hours of incubation.

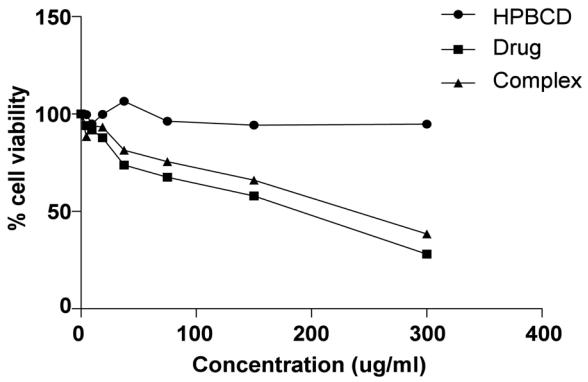

Figure 1: The effects of levodopa, HP-β-CD and the complex on relative cell viability of astrocytes cell line (CRL 2600) after 24 hours incubation period. Values represent mean (SD) from triplicate measurements.

Discussion

Glial cells represent the predominant constituent of human brain cells. Glial cells can be further categorised into two major groups, known as microglia and macroglia. The latter group includes ependymal cells, Schwann cells, oligodendroglia and astroglia. Alternatively, microglia include astrocytes, marginal glia, radial glia, cerebellar Bergmann glia, retinal Müller cells, neurohypophyseal pituicytes and hypothalamic tanycytes (14). In the brain defence system, astrocytes serve as a potent first line of defence by regulating the volume and composition of the extracellular space. Astrocytes are also important for maintaining normal brain functions, including the survival and migration of neurons during development. Moreover, astrocytes are critically involved in maintaining and regulating the trafficking across the blood brain barrier. In addition, astrocytes generate vasoactive metabolites in response to synaptic glutamate to meet the neuronal metabolic demand (15–17).

Although LD has been broadly used for the treatment of parkinsonism worldwide for years,

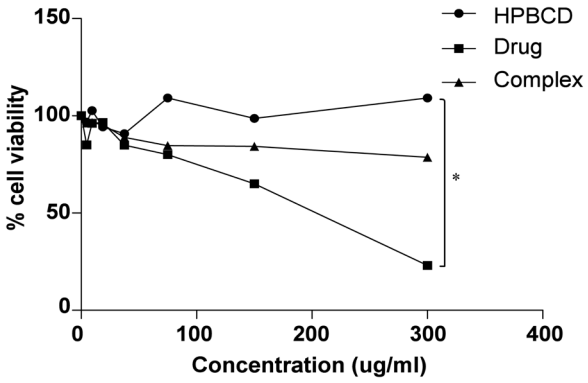

Figure 2: The effects of levodopa, HP-β-CD and the complex on relative cell viability of astrocytes cell line (CRL 2600) after 48 hours incubation period. Values represent mean (SD) from triplicate measurements.
*Statistically significant ($P < 0.05$) between HP-β-CD-treated group vs drug-treated group.

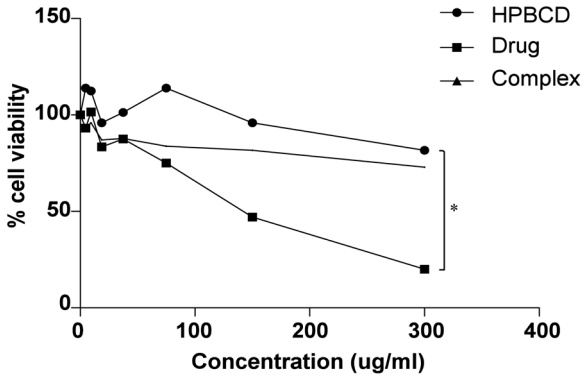

Figure 3: The effects of levodopa, HP-β-CD and the complex on relative cell viability of astrocytes cell line (CRL 2600) after 72 hours incubation period. Values represent mean (SD) from triplicate measurements.
*Statistically significant ($P < 0.05$) between HP-β-CD-treated group vs drug-treated group.

the potential toxicity of the drug itself and its potential contribution to neurodegeneration remains controversial. Such possibilities are based on the formation of oxygen free radicals and other reactive oxygen species due to the autoxidation of LD and DA. This phenomenon can destroy the lipid substructure of cell membranes, among other affects which might cause cell death. The potential mechanisms underlying cell death may include mitochondrial respiratory chain dysfunction, apoptosis, necrosis and excitotoxicity (18–21). Our results suggested that high concentrations of LD (> 200 µg/mL) are toxic to astrocytes in a time-dependent manner. Consistent with our findings, Ishida et al. (2000), reported that repeated administration of a high dose of LD increased the production of the hydroxyl radical in the dopamine-denervated striatum, which reflects the neurotoxicity of LD at a high dose (22). Previous studies have also demonstrated the dose-dependent nature of the toxicity of LD. In fact, exposure of low doses of LD to foetal midbrain neuronal cultures increased the survival rate and improved the neurite extension of dopaminergic neurons (17).

In the present study, the astrocyte cell line demonstrated a relatively high tolerability to HP-β-CD. Previous studies have demonstrated that the cytotoxic effects of CDs vary depending on the type of CD, the dosage applied, and the cell type examined. Schönfelder et al. (2006), reported that β-CD and M-β-CD induced the caspase-dependent death of human keratinocytes, whereas α-CD and HP-β-CD did not induce apoptosis in this cell type (23). Hipler et al. (2006), found that CDs at concentrations of up to 0.1% (w/v) were not toxic to HaCaT keratinocytes (24). However, at higher concentrations of 0.5 and 1.0%, an anti-proliferative response was observed, which reflected the toxic effects of CDs. As shown in figure 1–3, the activation of astrocytes in response to a toxicant may increase the percentage of cell viability. In this activation event, defined as reactive gliosis, phenotypic alterations of the cells, including excessive production of structural proteins and intermediate filaments, cellular hypertrophy and cell proliferation, are observed (25). Similar findings were reported by Sofian et al., (2012) (26), who found an increase in the rate of cell proliferation in a human glial cell line following exposure to β-CD for 72 hours. These data strongly agreed with the results reported by Gürbay et al. (2007) (27).

Interestingly, we found that the tolerability of astrocytes to LD was improved upon complexation with HP-β-CD, as demonstrated by an apparent time-dependent increase in cell viability. This finding may suggest the potential ability of HP-β-CD to mask the toxic effects of LD to this astrocyte culture. This concept is very strongly supported by many studies that demonstrated the unique ability of CDs to improve the physicochemical properties of entrapped drug molecules, including enhanced chemical stability, which may reduce the toxic effects of the unmodified drugs (Rasheed et al., 2008) (28).

Conclusion

In conclusion, our findings suggested that LD is toxic to an astrocyte culture at high concentrations (> 200 µg/mL). However, the complexation of LD with HP-β-CD resulted in an enhanced tolerability. Although HP-β-CD has been clinically demonstrated to be well-tolerated by humans, a slightly higher concentration might induce irreversible cell death and, thus, should be avoided.

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Conflict of Interest

None.

Fund

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Authors' Contributions

Conception and design: SSS
Analysis and interpretation of the data, drafting of the article: ZMS
Critical revision of the article for the important intellectual content: JMA, HO, SAR
Final approval of the article, SAR
Provision of study materials or patient, administrative, technical or logistic support: JMA, SAR
Collection and assembly of data: ZMS, SSS

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