Cytotoxicity evaluation of vancomycin and its complex with beta-cyclodextrin on human glial cell line

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Abstract: The possible cytotoxic effects of vancomycin and its complex with beta-cyclodextrin (β-CD) on human glial cell line (CRL 8621) were studied accordingly by means of MTS assay. The cultured cells were incubated with various concentrations of vancomycin, β-CD as well as β-CD/vancomycin complex ranging from 4.69 to 300 ug/ml. A linear dose-dependency cytotoxicity followed by hermetic-like biphasic dose-dependence was observed after incubation period of 72 hours. In general, significant increase (p<0.001) of cell proliferation was observed at lower concentrations: ≤18.75 µg/ml for cells treated with β-CD and their complex while ≤ 9.38 µg/ml for cells treated with vancomycin. In contrary, regardless of the treatments given, significant (p<0.001) reduce in cell survival was found at higher concentrations ≥150 µg/ml. In particular, 50 % inhibitory *in vitro* was achieved at the concentrations of 115.95 µg/ml (for β-CD), 116.48 µg/ml (for vancomycin) and 115.44 µg/ml (for β-CD/vancomycin complex).

Keywords: Cyclodextrin, vancomycin, glial, cytotoxicity.

INTRODUCTION

Vancomycin is a clinically important and probably the most studied amphotheric gylcopeptide antibiotic. It is primarily effective against serious infections caused by gram-positive cocci which are the most common isolated microorganisms from the brain infected areas (Backes *et al*., 1998; Tonon *et al*., 2006). Vancomycin inhibits the biosynthesis of bacterial cell wall by bindings to polypeptide intermediates terminating –D-Ala-D-Ala (Loll and Axelsen, 2000). Due to its time-dependent antibacterial activity and short half-life in cerebrospinal fluid (CSF), frequent administrations of vancomycin at high doses are required to maintain an appropriate serum level. Nephrotoxicity is the most frequent adverse effects associated with the administration of vancomycin and in more severe cases, hypotension can be observed (Bailie and Neal, 1988; Polk *et al*., 1993).

Over the last few decades, CDs have attained much attention in the pharmaceutical and drug formulations areas owing to their unique capability to form inclusion complex with a wide range of molecules ranging from solids, liquids and gases (Schmid, 1989). CDs are characterized as crystalline, homogenous, non-hygroscopic substances with a truncated cone shaped. The interior cavity of CDs is relatively hydrophobic while the external faces were found to be hydrophilic (Hirlekar and Kadam, 2008). Complexation with CDs will result in enhanced physicochemical properties of the entrapped guest molecules which include increased in solubility for poorly soluble guests, controlled volatility and sublimation as well as controlled release of the drugs (Szetjli, 2004; Wang *et al*., 2007).

Glial cells, classified as astrocytes, oligodendrocytes and microglia constitute 90% of the cells in our brain (Haydon, 2001). To date, the role of glial cells in integrity and degeneration of the central nervous system (CNS) has not been well elucidated. Nonetheless, studies have shown that glial cells are vital for neuronal survival. Astrocytes were shown to possess receptors including signaling molecules which are able to trigger neuronal massages that are keys to cell survival or death (Kimelberg and Nedergaard, 2010). In addition, there is evidence which demonstrated that astrocytes become activated in response to CNS pathologies with altered phenotypes (O'Callaghan *et al*., 1995).

Previously in our laboratory, the complexation of vancomycin with β-CD has been confirmed via several analytical chemistry methods including FTIR, SEM, TGA XRD and HPLC (data not shown). From the experiment, we found that the delivery of vancomycin was significantly enhanced (p<0.001) upon complexation with β-CD. In this study, a further investigation on the cytotoxic effects of the three compounds namely vancomycin, β-CD and their complex on human glial cell line was conducted accordingly by means of MTS assay.

MATERIALS AND METHODS

Materials

Vancomycin was purchased from Cheil Jedang, Korea. B-CD, phosphate-buffered saline (PBS) and dimethyl

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sulfoxide (DMSO) were obtained from Sigma-Aldrich, USA. The human glial cell line used was from the American Type Culture Collection (ATCC) (USA), Cat. no. CRL 8621. Eagle's Minimal Essential Medium (EMEM) supplemented with 10% heat inactivated fetal calf serum (FCS) was obtained from Mediatech, USA. 100U of penicillin/streptomycin and trypsin-ethyl-enediaminetetraacetic acid (EDTA) (0.05% trypsin and 0.53 mM EDTA-4Na) were purchased from Gibco-BRL, USA. (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) used was purchased from Promega, USA.

Cell culture

The cells were cultured in the sterile filter cap cell culture flasks with the growth areas of 25 cm^2 (Greiner Bio-One, Austria) and incubated in a humidified 5% of CO_2 at 37°C. They were grown in the EMEM supplemented with 10% heat inactivated FCS and 100U of penicillin/ streptomycin. When the cultures reached confluence, the adherent cells were detached by the following protocol: the cells were washed twice with sterile PBS and incubated with trypsin-EDTA for approximately 10 minutes at 37°C. The pellet was then collected by centrifugation at 1000 rpm, 4°C for 8 minutes. Cell counting was done by using an automated cell counter (Invitrogen, China). Dissociated cells were plated at the density of 5×10^4 viable cells/cm^2 in the medium. The medium was changed 24 hours after seeding.

Cytotoxicity study

For cytotoxicity study, vancomycin, β-CD and also the complex were added to the cells in seven different doses to achieve final concentrations ranging from 4.69 to 300 ug/ml. The cells were incubated for 24, 48 and 72 hours at 37°C. The optical density (OD) reading was taken using ELISA reader (LX 800). All vancomycin, β-CD and also the complex solutions were freshly prepared prior to analysis and were covered with aluminium foil to protect from light. The non-treated group was used as control. Cytotoxicity was assayed by MTS method (Malich *et al*., 1997).

STATISTICAL ANALYSIS

The one-way ANOVA test followed by a Tukey's test (SPSS 18) was performed to identify statistical significances. *p*-value <0.05 was considered to be statistically significant. IC_{50} values were obtained from dose-dependent curve plotted by Origin 8.0.

RESULTS

As shown in figs. 1-3, MTS assay of the treated glial cells with β-CD, vancomycin and β-CD/vancomycin complex ranging from 4.69-300 µg/ml demonstrated different cytotoxicity profiles depending on the treatments given and also the incubation periods. From fig. 1, general trend of linear dose-dependency cytotoxicity was observed for cells treated with β-CD for incubation periods of 24 and 48 hours. However, survival profile of the cells at 72 hours was found to be biphasic in nature. Significant (p<0.001) increase of cell proliferation was noted at lower concentrations: 4.69-18.75 µg/ml. At higher concentrations, cell proliferation was gradually decreased. Linear dose-dependency cytotoxicity was also observed upon subjecting cells to vancomycin and β-CD/vancomycin complex at 24 hours of incubation period. Low concentrations of vancomycin (≤ 9.38 µg/ml for 48 hours and 4.69 µg/ml for 72 hours) resulted in significant

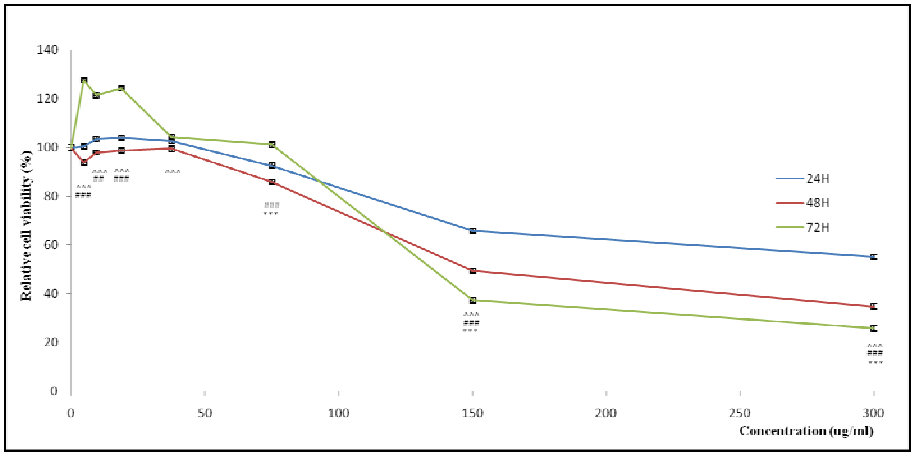

Fig. 1: The effect of beta-cyclodextrin on relative cell viability of human glial cell line (CRL 8621) after 24, 48 and 72 hours of treatments. Percentage of relative cell viability was determined by MTS assay. Values are mean ± S.D. of experiments performed in triplicate.

*** Statistically significant difference ($p < 0.001$) between the treatment doses vs. non-treated after 24 hours

Statistically significant difference ($p < 0.001$) between the treatment doses vs. non-treated after 48 hours

^^^ Statistically significant difference ($p < 0.001$) between the treatment doses vs. non-treated after 72 hours

Statistically significant difference ($p < 0.01$) between the treatment dose vs. non-treated after 48 hours

(p<0.001) increase of cell viability. Similar results were obtained for cells treated with β-CD/vancomycin complex from 4.69-18.75 µg/ml at 48 hours and from 4.69-75 µg/ml at 72 hours. In particular, we found that 50% inhibitory *in vitro* was achieved at the concentrations of 115.95 µg/ml (for β-CD), 116.48 µg/ml (for vancomycin) and 115.44 µg/ml (for β-CD/vancomycin complex) (figs. 4-6). Statistically, from fig. 7, no significant difference was quantified between the IC_{50} values calculated.

DISCUSSION

Studies on the cytotoxic effects of CDs and vancomycin on various cells have been reported before. Nonetheless, to the best of our knowledge, similar studies on human glial cells as presented in our report remained unexplored. In this present study, we found that glial cells responses to β-CD, vancomycin and also their complex were time- and dose-dependent. For cells treated with β-CD, a linear dose-dependent cytotoxicity was observed at incubation period of 24 and 48 hours. Similar response was also obtained by subjecting cells to vancomycin and β-CD/vancomycin complex at 24 hours of incubation period. On the other hand, at ≥ 48 hours (for cells treated with vancomycin and β-CD/vancomycin complex) and at 72 hours (for cells treated with β-CD) of incubation

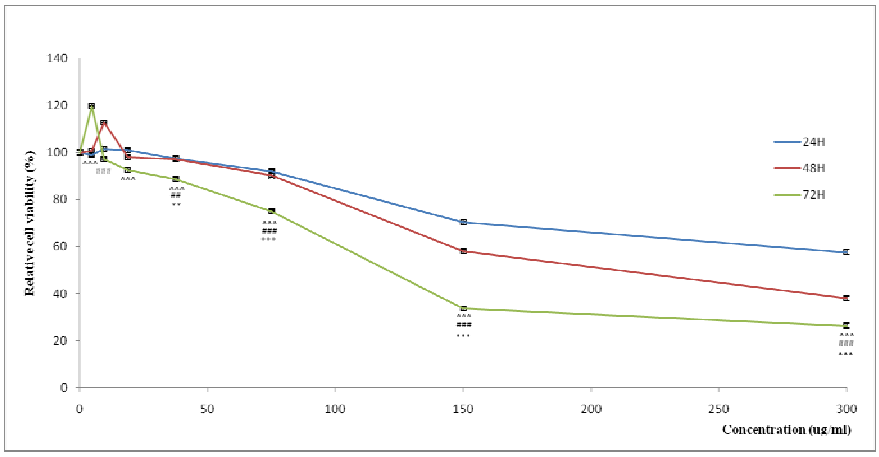

Fig. 2: The effect of vancomycin on relative cell viability of human glial cell line (CRL 8621) after 24, 48 and 72 hours of treatments. Percentage of relative cell viability was determined by MTS assay. Values are mean ± S.D. of experiments performed in triplicate.
*** Statistically significant difference ($p < 0.001$) between the treatment doses vs. non-treated after 24 hours
Statistically significant difference ($p < 0.001$) between the treatment doses vs. non-treated after 48 hours
^^^ Statistically significant difference ($p < 0.001$) between the treatment doses vs. non-treated after 72 hours
** Statistically significant difference ($p < 0.01$) between the treatment dose vs. non-treated 24 hours
Statistically significant difference ($p < 0.01$) between the treatment dose vs. non-treated 48 hours

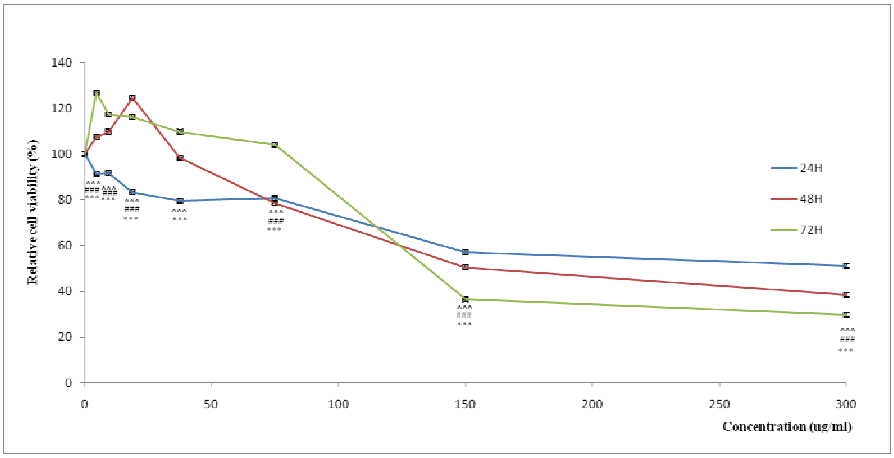

Fig. 3: The effect of beta-cyclodextrin/vancomycin complex on relative cell viability of human glial cell line (CRL 8621) after 24, 48 and 72 hours of treatments. Percentage of relative cell viability was determined by MTS assay. Values are mean ± S.D. of experiments performed in triplicate.
*** Statistically significant difference ($p < 0.001$) between the treatment doses vs. non-treated after 24 hours
Statistically significant difference ($p < 0.001$) between the treatment doses vs. non-treated after 48 hours
^^^ Statistically significant difference ($p < 0.001$) between the treatment doses vs. non-treated after 72 hours

period, hermetic-like biphasic dose-responses were recorded. In general, significant (p<0.001) increase of cell proliferation was observed at lower concentrations: ≤18.75 µg/ml (0.0018%, w/v) for cells treated with β-CD and their complex while ≤ 9.38 µg/ml (0.0009 %, w/v) for cells treated with vancomycin. In contrary, regardless of the treatments given, significant (p<0.001) reduce in cell survival was found at higher concentrations ≥150 µg/ml (0.015 %, w/v).

Relative to other studies, we found that the cytotoxic effects of β-CD and vancomycin varied depending on the type of cells used. Piel *et al* (2004) reported that no damage was found on human stratum corneum even at high concentration of 5% (w/v) CDs at 2 hours incubation period. In other study reported by Hipler *et al* (2006), it was found that HaCaT kerationocytes cells exposed up 0.1% (w/v) of CDs did not show any anti-proliferative response but at higher concentrations, 0.5 and 1.0 % (w/v) respectively, negative proliferation was noted indicating the cytotoxic effects exerted by CDs. As for vancomycin, a study conducted by King and Smith (2004) revealed that vancomycin exposure (1, 2.5 and 5 mM) induced cell proliferative response in renal proximal tubule cells (LLCPK1) by mean of dose- and time-dependent manners. However, a report by Yoeruek *et al*. (2008) suggested that high concentration, 15 mg/ml (~10 mM) of vancomycin was shown to significantly reduce in viability of human corneal endothelial cells (HCEC) as predomination of late cell apoptotic was observed.

From our results, increase in cell survival at lower concentrations may originate from the activation of astrocytes during the process known as reactive gliosis. Astrocytes become activated in response to toxicants. In such event, phenotype alterations of astrocytes included enhanced production of structural protein glial fibrially acidic protein and intermediate filaments, cellular

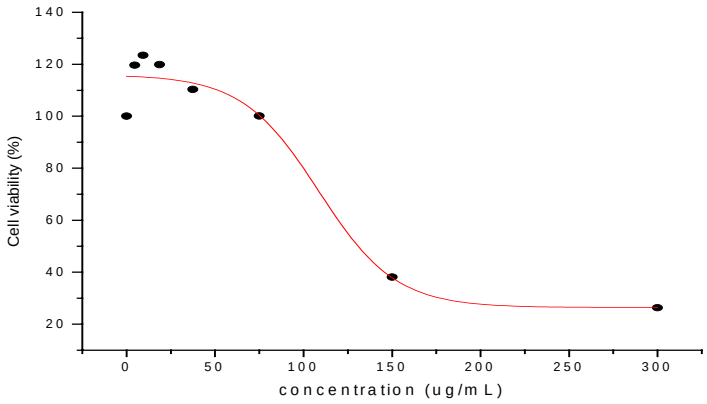

Fig. 4: IC_{50} dose dependent curve for glial cells treated with beta-cyclodextrin. Values are mean from triplicate measurements (Origin 8.0).

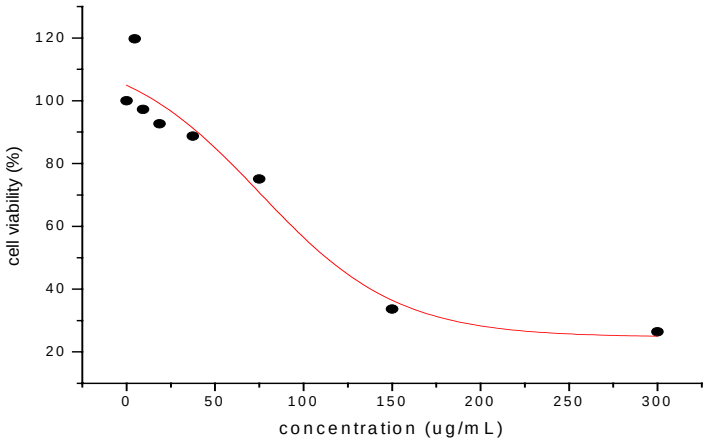

Fig. 5: IC_{50} dose dependent curve for glial cells treated with vancomycin. Values are mean from triplicate measurements (Origin 8.0).

hypertrophy and sometimes cell proliferation could be seen (O'Callaghan *et al*., 1995). Similar finding was also claimed by Gürbay *et al*. (2007) following 24 hours incubation of rat astrocytes with ciprofloxacin (CFX).

The ability of β-CD-induced cytotoxic effect at high concentrations may be due to its high binding affinity towards cholesterol. Cholesterol efflux was found as high as 31% after 2 hours incubation of 2.5 mM β-CD in brain capillary endothelial cells (BCEC). In support to their findings, Kilsdonk *et al* (1995) reported that about 50-90% of cholesterol in L-cells, Fu5AH-cells and fibroblast was dissolved upon 8 hours incubation with β-CDs. High level of cholesterol and sphingolipids were found in the lipid rafts, the microdomains inside the exoplasmatic leaf of the lipid bilayer (Simons and Ikonen, 1997). Previous findings suggested that cholesterol was involved in signal transduction processes in addition to its role in ensuring the physical stability of plasma membranes. Thus, significant depletion of cholesterol level will lead in destroying the integrity of the lipid grafts which ultimately brings major changes in the signal transduction cascades (Rufini *et al*., 2009; Borisova *et al*., 2010).

Toyoguchi *et al* (2010) and Horinouchi *et al* (1993) revealed that vancomycin provoked release of histamine

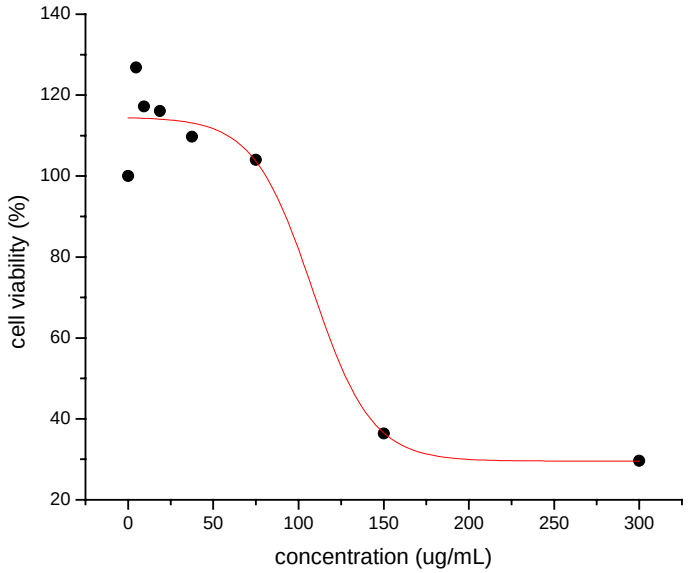

Fig. 6: IC_{50} dose dependent curve for glial cells treated with beta-cyclodextrin/vancomycin complex. Values are mean from triplicate measurements (Origin 8.0).

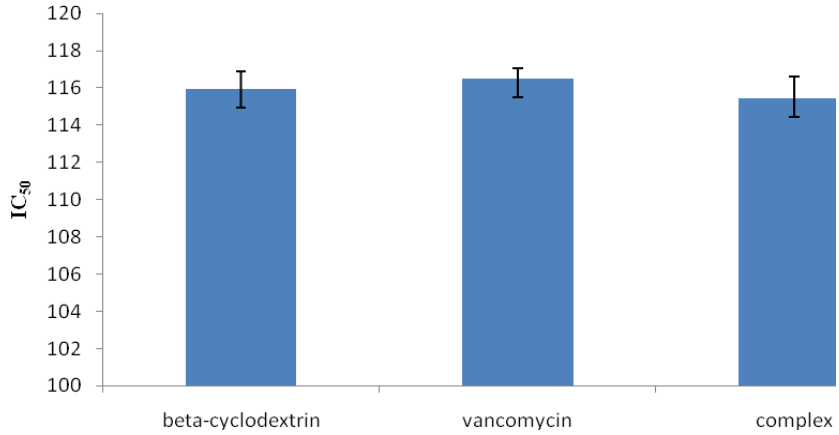

Fig. 7: IC_{50} values calculated for treated groups. Values are the mean ± S.D. of experiments performed in trplicate (One-way ANOVA). Values were calcuted using Origin 8.0.

in dose-dependent manner in rat peritoneal mast cells. High level of histamine (1×10^{-4} mol/L) released was found to inhibit the proliferation of human keratinocytes cells (HKC) while at low concentration (1×10^{-8} mol/L) promoted cell proliferation. It was also reported in the same study, histamine at high concentrations inhibited the progress of HKC cell cycle, mediated cell apoptosis and induced the increased of [Ca^{2+}] (Ran *et al*., 2005). These findings appeared to be consistent with our results as low concentrations of vancomycin were found to induce cell proliferation but significantly reduce cell survival at high concentrations. The homeostasis of [Ca^{2+}] is vital for development and survival of virtually all types of cells including glial cells in which overload of [Ca^{2+}] resulted from dysregulation of channels and pumps will ultimately lead to deleterious of glial cells (Simpson *et al*., 1998). Nonetheless, the specific mechanisms of β-CD- and vancomycin- induced cytotoxicity in human glial cells remained elusive and yet to be studied.

CONCLUSION

In conclusion, our results suggested that β-CD, vancomycin and their complex triggered a linear dose-dependent cytotoxicity followed by hermetic-like biphasic dose-dependence in human glial cells *in vitro* following 72 hours of incubation period which may result from the complex dose-dependent relationships between glial cells viability and inherent properties of glial cells. Although β-CD and vancomycin are clinically proven to be well tolerated in human, slightly higher concentrations might induce irreversible cell death and thus should be avoided.

However, due to the capability of β-CD to deliver vancomycin over a prolonged period of time with minimal cytotoxic effects as proven in our study renders this complex as a promising product to be used in the future for site-specific administration of vancomycin particularly for the treatment of localized Gram-positive bacterial infections in CNS.

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