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Research Report

ANTIVIRAL ACTIVITY OF COPPER(II)CHLORIDE DIHYDRATE AGAINST DENGUE VIRUS TYPE-2 IN VERO CELL

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ABSTRACT

Infection of dengue virus (DENV) was number of globally significant emerging pathogen. Antiviral dengue therapies are importantly needed to control emerging dengue. Dengue virus (DENV) is mosquito-borne arboviruses responsible for causing acute systemic diseases and grievous health conditions in humans. To date, there is no clinically approved dengue vaccine or antiviral for humans, even though there have been great efforts towards this end. Copper and copper compounds have more effective in inactivation viruses, likes an influenza virus and human immunodeficiency virus (HIV). Purpose in this project was investigated of Copper(II) chloride Dihydrate antiviral compound were further tested for inhibitory effect on the replication of DENV-2 in cell culture. DENV replication was measures by Enzyme-linked Immunosorbent Assay (ELISA) with selectivity index value (SI) was determined as the ratio of cytotoxic concentration 50 (CC_{50}) to inhibitory concentration 50 (IC_{50}) for compound. The maximal inhibitory concentration (IC_{50}) of Copper(II)chloride Dihydrate against dengue virus type-2 was 0.13 µg/ml. The cytotoxic concentration (CC_{50}) of compound against Vero cell was 5.03 µg/ml. The SI values for Copper(II)chloride Dihydrate 38.69. Result of this study suggest that Copper(II) chloride Dihydrate demonstated significant anti-DENV-2 inhibitory activities and not toxic in the Vero cells. Copper mechanisms play an important role in the prevention of copper toxicity, exposure to excessive levels of copper can result in a number of adverse health effects, as a result increased reactive oxygen species and oxidative damage to lipid, DNA, and proteins have been observed in human cell culture models or clinical syndromes of severe copper deficiency and inhibition was attributed to released cupric ions which react with cysteine residues on the surface of the protease.

Keywords: *antiviral, dengue virus type-2, Copper(II)chloride Dihydrate, inhibitory, cytotoxity*

ABSTRAK

Infeksi virus dengue (DENV) adalah patogen yang muncul secara global. Terapi antivirus dengue penting diperlukan untuk mengontrol muncul dengue. Dengue virus (DENV) disebabkan oleh mosquito-borne arboviruses yang menyebabkan penyakit sistemik akut dan kondisi kesehatan pada manusia. Sampai saat ini, tidak ada vaksin dengue klinis disetujui atau antivirus bagi manusia, meskipun telah ada upaya besar menjelang akhir ini. Tembaga dan senyawa tembaga memiliki efektivitas dalam inaktivasi virus, seperti virus influenza dan human immunodeficiency virus (HIV). Tujuan dalam proyek ini adalah menyelidiki senyawa antiviral Copper (II) klorida Dihidrat yang kemudian diuji lebih lanjut untuk efek penghambatan pada replikasi DENV-2 dalam kultur sel. Replikasi DENV diukur dengan Enzyme-linked Immunosorbent Assay (ELISA) dengan nilai indeks selektivitas (SI) ditentukan sebagai rasio konsentrasi toksisitas 50 (CC_{50}) ke konsentrasi penghambatan 50 (IC_{50}) senyawa. Maksimal konsentrasi penghambatan (IC_{50}) Tembaga(II)klorida Dihidrat terhadap virus dengue tipe 2 adalah 0,13 µg/ml. Konsentrasi toksisitas (CC_{50}) senyawa terhadap sel Vero adalah 5.03 µg/ml. SI nilai untuk Tembaga(II)klorida Dihidrat 38.69. Hasil penelitian ini menunjukkan bahwa Tembaga(II) klorida Dihidrat signifikan anti-DENV-2 dan tidak toksik dengan sel Vero. Mekanisme tembaga berperan penting dalam pencegahan toksisitas tembaga, paparan kadar tembaga yang berlebihan dapat mengakibatkan sejumlah efek kesehatan yang merugikan, akibatnya

peningkatan spesies oksigen reaktif dan kerusakan oksidatif pada lipid, DNA, dan protein telah diamati pada Model kultur sel manusia atau sindrom klinis defisiensi tembaga berat dan penghambatan dikaitkan dengan ion cuprik yang dikeluarkan yang bereaksi dengan residu sistein pada permukaan protease.

Kata kunci: *antivirus, virus dengue tipe-2, Tembaga(II)klorida Dihidrat, penghambatan, toksisitas*

INTRODUCTION

Infection of dengue virus (DENV) was number of globally significant emerging pathogen. It is member of Flaviviridae family, with the genus Flavivirus. DENVs were distributed in the tropical and sub-tropical areas and transmitted to humans by Aedes agypty and Aedes albopictus.[1] Dengue virus (DENV) is mosquito-borne arboviruses responsible for causing acute systemic diseases and grievous health conditions in humans. More than 2.5 billion cases of dengue infection occurred in the worldwide.[2] Indonesia is one of the largest counties in the dengue endemic region worldwide. Dengue was occurred for the first time as an outbreak in Surabaya and Jakarta in 1968.[3] To date there are not effective vaccine and antiviral treatment for DENV, patient supportively-treated without any specific treatment measures.[4] Antiviral dengue therapies are importantly needed to control emerging dengue. Effective antiviral therapies, currently unavailable for any type of DENV, are urgently needed to ameliorate the disease burden by DENV.[5] Ribavirin has shown activity against all flaviviruses tested in a broad array of cell types in vitro but efficacy in vivo has generally been poor, ribavirin can be toxic in vivo.[6] A compound that exhibited a lower effective dose and toxicity than ribavirin while retaining its broad spectrum of activity would be particularly desirable as a candidate flavivirus therapy.[5]

Copper and copper compounds have been used as important antiviral material.[7] Recently, group found that Cu^{+} species in the related compounds is much more effective in inactivation of bacterial and viruses than copper metal and copper(II) compounds.[8] On the other hand, copper has long been used as an antibacterial material,[9] and several copper compounds have been reported to exhibit viral inactivation. More recently, the inactivation of avian influenza virus by copper metal[10] and divalent ions (Cu^{2+})[11] and the inactivation of human immunodeficiency virus (HIV) by copper ions[12] and copper oxide have been reported.[13] Copper iodide nanoparticle against for feline calicivirus (FCV) was demonstrated that the antiviral behaviors of CuI nanoparticles against FCV were identified to detect Cu^{+} ions, hydroxyl radicals, and capsid protein oxidation. Copper iodide nanoparticles showed high antiviral activity against FCV was attributed to Cu^{+} ions, followed by ROS (O_2• or •OH) generation and subsequent capsid protein oxidation.[14] The antivirus properties of the $CuFeO_2$ crystals achieved about 8 log inactivation of the phage after 4 h of contact time in the dark, $CuFeO_2$ are good chemical stability in a weak acid condition.[7] Copper is a bio-essential element and copper complexes have been extensively utilized in metal mediated DNA cleavage for generation of activated oxygen species, was reported that teraaza macrocyclic copper coordination compounds have anti-HIV activity. Macrocyclic complexes can react with DNA in different binding fashions and axhibit effective nucleus activities.[15]

Copper monodispersed nanoparticles (2-5 nm) in submicron particles of sepiolite, structure of sepiolite is $Mg_8Si_{12}O_{30}(OH)_4(H_2O)_4.8H_2O$, have revealed as a strong bactericide so that they were able to decrease the starting microorganism concentrations of Staphylococcus aureus or Escherichia coli by 99.9%.[16] The antibacterial stainless steels included a copper-bearing austenitic antibacterial showed excellent with antibacterial rate to E.coli over 99.99%, copper ions play the dominant role in the antibacterial effect of antibacterial stainless steels acted with E. coli.[17]

Previous result, ribavirin exerts its toxicity through an inhibiton of intracellular energy metabolism and axidative membrane damage, leading to an accelerated extravascular hemolysis by the reticulo-endothelial. But not significant inhibiton of level, ribavirin was more to toxic to replicating cells than to stationary cell monolayers in Vero cells.[18] Currently, there is no published data on the possible anti-DENV activities of Copper and copper compounds. In the present study, we investigated of Copper(II)chloride Dihydrate antiviral compound were futher tested for inhibitory effect on the replication of DENV-2 in cell culture.

MATERIAL AND METHOD

Material

Chemical reagents used in this research is the Copper(II) chloride Dihydrate ($CuCl_2.2H_2O$) (Merck 99.0%), Dimethyl Sulfoxide (DMSO) (Merck 99.98%), Minimum Essential Medium Eagle (MEM Media) (Sigma-Aldrich), DENV-2 Surabaya Isolate, Vero cell (African green monkey kidney), Cell Proliferation Reagent WST-1 (Roche Applied Science), and Dengue Virus Antibody (4G2) for ELISA.

Method

Antiviral activity assay

Confluent monolayers of Vero cells were prepared in 96 wells cell culture microplate. The numbers of DENV-2 were counted using a Hemocytometer and the titer of virus was expressed as Foci-Forming-Unit (FFU). Seed Vero cells in a 96-well plate ($1x10^6$ cells/10 ml), add serially diluted

test compounds to Vero cells, add DENV-2 solution ($2x10^4$ FFU/well) and incubate 37°C for 2 days. The percentage of inhibition concentration (IC_{50}) compared with controls was calculated as follows: IC_{50} (%) = (NC-AC) x 100/ NC. Where, NC is the mean of the number for negative control and AC is the number absorbance of compound. Inhibition of compound to DENV-2 was further verified using quantitative Enzyme-linked Immunosorbent Assay (ELISA).

Cytotoxicity assay

Cytotoxicity used WST-1 cell proliferation reagent by Roche Applied Science, Mannheim, Germany.[19] The dye of WST-1 reagent has a larger linear range and increased stability compared to other tetrazolium salt based assays. The WST-1 assay is suitable for use with adherent and suspension cells. The assay is very sensitive, it can detect 500 to 50,000 cells in a single well of a 96-well plate. Vero cells ($1x10^5$ cells/ml) were seeded in 96-well plate at 37 °C in 5% CO_2 overnight. A total of 100 µl of serial delusion compound were incubated with Vero cells for 24 h. A total of 10 µl of Cell Proliferation Reagent WST-1 was added into each well, incubated for 1 hour at 37 °C. The plate was read at 450 nm (main filter) and 655 nm (reference filter) using an ELISA reader (iMarkTM Microplate Absorbance Reader).

RESULT AND DISSCUSSION

Inhibitory Effect of Copper(II)chloride Dihydrate

Copper(II)chloride Dihydrate were futher studied for their inhibitory effect on replication of the DENV-2 in Vero cells. The IC_{50} (inhibitory concentration 50) was determined from the dose response curves. This compound proved to be effective as inhibitor of replication of DENV-2, the IC_{50} value was 0.13 µg/ml and R^2 value was 0.9812 with selectivity indices (SI) was 38.69. The selectivity indices of these antiviral compounds appeared to be moderately influenced by the strain of DENV tested.

The mechanisms of how Copper(II)chloride Dihydrate exerts it is anti DENV-2 effects are not known. However, the effects of other compounds against cellular RNA polymerases and formation of the complex with RNA have been reported suggesting that Copper(II)chloride Dihydrate could also affect the similar replication enzymes. Viral replication was inhibited during a simultaneous treatment assay, indicating that the entry of the virus was impeded by peptide.

Previous research was reported protease inhibitory activity on DENV2V NS3 target, Palmatine has active concentration 26.4 µM, this compound was subsequently analyzed for antiviral activity in cell-based replication assays in cell culture.[20] The neutral red assay mean EC_{50} of ribavirin was only 106 µg/ml with SI of 9.4 against West Nile Virus (WNV) New York isolate and EC_{50} of

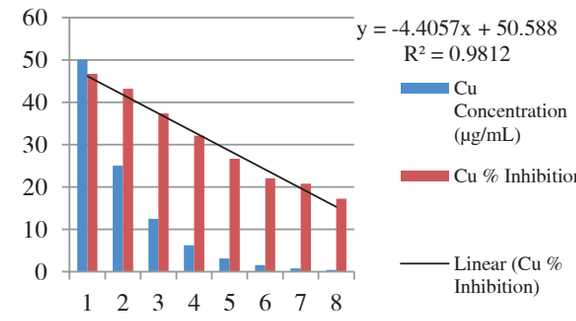

Figure 1. Inhibitory chart of Copper(II)chloride Dihydrate for Vero cells by ELISA

6-Azauridine was 1.5 µg/ml with SI of WNV New York isolate. This result confirm by virus yield reduction assay when the assay when the assay was performed 2 days after initial infection in Vero cells.[21]

Copper homeostatic mechanisms play an important role in the prevention of copper toxicity, exposure to excessive levels of copper can result in a number of adverse health effects. Similar to Cu toxicity, Cu deficiency also affects, directly or indirectly, the components of the oxidant defense system and as a result increased reactive oxygen species and oxidative damage to lipid, DNA, and proteins have been observed in human cell culture models or clinical syndromes of severe copper deficiency.[22] In these cases, the observed inhibition was attributed to released cupric ions which react with cysteine residues on the surface of the protease.[23]

Maximal inhibitory concentration (IC_{50}) of quercetin against DENV-2 was 35.7 µg/ml when it was used after virus absorption to the cells and decreased to 28.9 µg/ml when the cells were treated continuously for 5 h before virus infection and up to 4 days post-infection. A weak effect for prophylactic activity of quercetin however. These findings suggest that the main anti-dengue activity of quercetin is likely due to its activity against the different stages of its replication of DENV-2 instead of early stages of intracellular replication cycle such as virus attachment or entry.[4]

Cytotoxicity of Copper(II)chloride Dihydrate

The cytotoxicity study was carried out for compound of Copper(II)chloride Dihydrate. This extract was screened for its cytotoxicity against Vero cells at different concentrations to determine the CC_{50} by WST-1 assay.

The percentage growth cytotoxicity was found to be increasing with increasing concentration of test compound, and that show in figure 4. Copper(II)chloride Dihydrate effect on Vero cells (CC_{50}) up to 5.03 µg/ml and R^2 value was 0.9174. In this work, we have examined the relationship between the concentration in the culture medium of Vero cells and the cytotoxic potency of Copper(II)chloride Dihydrate.

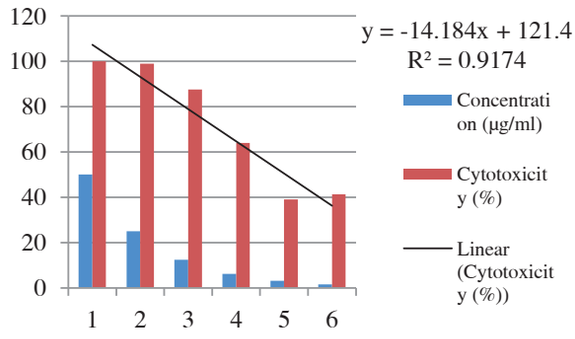

Figure 2. Cytotoxicity chart of Copper(II)chloride Dihydrate for Vero cells by WST-1 assay

CONCLUSION

As the conclusion, the study demonstrated that the Copper(II)chloride Dihydrate exhibited significant anti DENV-2 replication properties. These results suggest that these Copper(II)chloride Dihydrate could be further investigated IC_{50} was 0.13 µg/ml, CC_{50} was 5.03 µg/ml, and SI was 38.69.

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