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Protection of Retinal Cells from Ischemia by a Novel Gap Junction Inhibitor

Satyabrata Das^a, Dingbo Lin^a, Snehalata Jena^a, Aibin Shi^b, Srinivas Battina^b, Duy H. Hua^b, Rachel Allbaugh^c, and Dolores J. Takemoto^{a,*}

^a Department of Biochemistry, Kansas State University, Manhattan, KS 66506, USA

^b Department of Chemistry, Kansas State University, Manhattan, KS 66506, USA

^c Vet Med Teaching Hospital, Kansas State University, Manhattan, KS 66506, USA

Abstract

Retinal cells which become ischemic will pass apoptotic signal to adjacent cells, resulting in the spread of damage. This occurs through open gap junctions. A class of novel drugs, based on primaquine (PQ), was tested for binding to connexin 43 using simulated docking studies. A novel drug has been synthesized and tested for inhibition of gap junction activity using R28 neuro-retinal cells in culture. Four drugs were initially compared to mefloquine, a known gap junction inhibitor. The drug with optimal inhibitory activity, PQ1, was tested for inhibition and was found to inhibit dye transfer by 70% at 10 μ M. Retinal ischemia was produced in R28 cells using cobalt chloride as a chemical agent. This resulted in activation of caspase-3 which was prevented by the PQ1 gap junction inhibitor. Results demonstrate that novel gap junction inhibitors may provide a means to prevent retinal damage during ischemia.

Keywords

Ischemia; Cobalt Chloride (CoCl_2); Gap junctions; Retinal degeneration; Hypoxia; Caspase-3; HIF1- α ; PQ1

Introduction

Retinal ischemia is a major cause of vision loss in various retinal diseases, e.g., diabetic retinopathy, glaucoma, and stroke [1–3]. The apoptosis of retinal neurosensory cells, which occurs during ischemia, is thought to be through open gap junctions, propagated by the “bystander effect” [4].

Gap junctions are hydrophilic channels and/or hemichannels that allow the passage of both necessary metabolites and apoptotic signals from cell to cell [5–9]. The gap junction “bystander effect” occurs when a dying cell delivers a cellular apoptotic signal such as high Ca^{+2} or ATP to an adjacent cell through open gap junctions which, in turn, causes spread of the death signal [4]. The process is well-documented in brain ischemia [10–12]. Retinal ischemia induces

*Corresponding author: Dolores J. Takemoto, Department of Biochemistry, Kansas State University, 141 Chalmers Hall, Manhattan, KS, 66506, Phone: +1 785-532-7009, Fax: +1 785-532-7278, E-mail address: dtak@ksu.edu (D.J. Takemoto).

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retinal cell death in a caspase-dependent manner [13]. We have previously found that inhibition of gap junctions, in hippocampal HT22 cells, prevents oxidative cell death due to H_2O_2 through a caspase-3 pathway [14]. It is apparent that proper control of gap junctions is essential for neural cell survival [15].

In the current study, we used cobalt chloride ($CoCl_2$) to induce a chemical hypoxia/ischemia condition in a rat retinal neurosensory cell line, R28. $CoCl_2$ has been shown to induce oxidative damage through the generation of reactive oxygen species (ROS) in a wide variety of cells [16] and has been recently reported to cause degeneration of mammalian retinal photoreceptor cells [17]. R28 cells offer a well-characterized population of precursors to multiple neuroretinal cell types to investigate ischemia-induced apoptosis.

In this study we determined the efficacy of a novel group of gap junction inhibitors which are based on primaquines. Previously the anti-malarial chloroquine drugs, such as mefloquine, have been reported to be potent inhibitors of the gap junction protein, Cx50 [18,19]. Based on simulated docking studies using this class of substituted quinolines, several structures were found to dock into the channel pore of Cx43. These drugs were synthesized and tested. The results suggest a novel class of gap junction inhibitors which can be used to prevent neural and retinal cell damage due to ischemia.

Materials and Methods

Cell cultures

The rat retinal neurosensory R28 cells were cultured in DMEM (low glucose) (Invitrogen, CA) supplemented with 10 % fetal bovine serum and 50 μ g/ml gentamicin, 50 units/ml penicillin, 50 μ g/ml streptomycin, pH 7.4 at 37°C in an atmosphere of 95 % air and 5 % CO_2 .

Design and Synthesis of primaquine-1 (PQ1)

In search of new inhibitors that inhibit gap junction intercellular communication (GJIC), we examined potential interactions of a number of substituted quinolines (code name PQs) with the partial crystal structure of the Cx43 hemichannel [20,21] using Autodock computational docking software [22–24]. In one of the minimum energy (–0.7 kcal/mol) bound structures, interactions (close contacts) between CF_3 group of PQ1 and H-N of Leu144 of connexin (2.5 Å), OCH_3 group of PQ1 and CH_2 of Phe81 of connexin (2.0 Å), and NH_3^+ of PQ1 and ^-O_2C -Glu146 of connexin were found. Consequently, we synthesized this class of quinolines and determined their ability to inhibit gap junction dye transfer. PQ1 was synthesized via a modification of the reported protocol [25–27] starting from 4-acetaminoanisole. The detailed synthesis is described in Ref. 31.

Cobalt chloride ($CoCl_2$) treatment-a chemical hypoxia model in R28 cells

Approximately 70 % confluent retinal R28 cells were pre-incubated with PQ1 (10 μ M, 40 min), followed by $CoCl_2$ treatments at 100, 200 and 500 μ M for different time periods in a cell culture chamber (5 % CO_2 , room air, 37°C). Hypoxia induction was confirmed by testing the hypoxia-inducible factor 1- α (HIF1 α) protein expression levels in nuclear extracts by Western blotting.

Gap Junction Activity Assay

R28 cells were grown to 90% confluency on coverslips. They were treated with PQ1 at 10 μ M for 40 minutes. A mixture of 1% each, in PBS, Lucifer Yellow (LY) and Rhodamine Dextran (RD) (Molecular Probes, Eugene, OR) were added to the cells at the center of the coverslip. Two cuts across the coverslip were made to form a transient tear in the plasma membranes of the cells to permit dye entry into cells. Cells were incubated with 2.5 μ l of both of the dyes for 20 minutes, then fixed in 2.5% paraformaldehyde, washed in PBS and examined by fluorescent

microscopy using a Nikon C1 confocal microscopy. For quantitation, the extent of dye transfer was calculated by counting the number of LY-labeled cells from the initial scrape with subtraction of RD-labeled cells, as a cell damage control, in the microscopic field. Four points per slide were photographed as previously described [28]. The experiments were repeated six times, and data are mean $\pm$ S.E.M.

Nuclear Extracts

Following treatments with PQ1 and/or CoCl₂, R28 cells were scraped into cold phosphate-buffered saline, centrifuged and washed once in five packed cell volume equivalents of buffer A (10mM Tris-HCl (pH 7.5), 1.5 mM MgCl₂, 10mM KCl) freshly supplemented with 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 0.4 mM phenylmethylsulfonyl fluoride, and 10 μ l/ml of protease inhibitor cocktail (Sigma # P8340). Cell pellets were resuspended in 2.5 packed cell volume equivalents of buffer A and incubated in a pre-chilled Dounce homogenizer on ice for 10 min followed by homogenization by 20 strokes with a type B pestle [29,30]. Nuclei were pelleted by centrifugation at 10,000 $\times$ g for 10 min, the supernatant was discarded and nuclei were resuspended in 3.5 packed nuclear equivalent volumes of buffer B (20mM Tris-HCl (pH 7.5), 1.5 mM MgCl₂, 0.42 M KCl, 20% (v/v) glycerol) freshly supplemented with 2mM DTT, 1 mM sodium orthovanadate, 0.4 mM phenylmethylsulfonyl fluoride, and 10 μ l/ml of protease inhibitor cocktail. The suspension was rotated at 4°C for 30 min and centrifuged for 30 min at 14,000 rpm. The eluted nuclear proteins in the supernatant were collected and HIF1 α protein levels were measured by western blotting.

Western blot

Western blotting was performed as described previously [28]. Anti-Cx43 was purchased from Fred Hutchinson cancer research center (# Cx43NT1), anti-HIF1- α was purchased from Novus Biologicals (# NB100–105), and anti-caspase-3 (# 9661), phospho-Cx43 (Ser368) (#3511S) were purchased from Cell Signaling Technology and anti- β -actin was purchased from Sigma (# A5441).

Apoptosis Assay

Approximately 70% confluent R28 Cells in 25 cm² flasks were treated with PQ1 at 10 μ M for 40 minutes followed by treatment with 500 μ M CoCl₂ for different time periods to induce apoptosis. After this, cells were harvested and stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's protocol (BioVision # K101–100). Annexin V-FITC/PI binding was analyzed by flow cytometry using a BD FACSCalibur system and data was analyzed using the CellQuest software.

Statistical analyses

All analyses represent at least triplicate experiments. The statistical analysis employed in this paper is the Student's *t*-test. The level of significance (*) was considered at $p \leq 0.05$. All data are mean $\pm$ S.E.M.

Results and Discussion

Docking and Synthesis of Primaquine 1 (PQ1)

Since selective inhibition of gap junction intercellular communication with small molecules may potentially prevent cells from death during ischemic stroke, we used computational docking methods to search for chemicals that bind to the Cx43 gap junction hemichannel, based upon the partial crystal structure. After screening several classes of molecules, we focused on substituted quinolines based on their relative binding constants and bioactivities. Primaquine 1 (PQ1) analogs were synthesized via a modification of the reported protocol [25–27] starting

from 4-acetaminoanisole [27]. The succinic acid salt of PQ1 (structure shown in Fig. 1A) was prepared to provide water-soluble material for biological evaluation. Succinic acid alone does not show bioactivities. The interaction between the NH_3^+ group (under physiological conditions, N^4 -amino function of PQ1 exists as protonated form) of PQ1 with the carboxylate ion (negatively charged) of Glu146 of the Cx43 may be significant. Docking studies are shown in Figs. 1B and C.

PQ1 inhibits gap junction activity in retinal R28 cells with similar efficacy when compared to mefloquine

During the synthesis of PQ1 several intermediates were tested and compared to mefloquine, a known gap junction inhibitor [18,19]. Gap junction dye transfer of Lucifer yellow was measured and results are shown in Fig. 2A and B. At 10 μM for 40 min, mefloquine (MQ) inhibited dye transfer by approximately 50% while PQ1, at the same dose inhibited dye transfer by 70%. The intermediates, PQ2 and PQ3 had poor inhibitory activity while PQ4 was similar to MQ. Since PQ1 had the greatest inhibitory activity and was water soluble, this drug was further tested for protection from ischemia-induced apoptosis.

PQ1 protects R28 cells from ischemic apoptosis induced by cobalt chloride (CoCl_2)

Next, we determined whether PQ1 inhibition of gap junctions could prevent retinal neurosensory R28 cells from apoptosis using a chemical (CoCl_2)-induced ischemia system as our model. As shown in Figure 3A, CoCl_2 incubation at 500 μM for 24 hours induced activation of caspase-3. Pre-incubation of R28 cells with PQ1 at 10 μM for 40 min followed by co-incubation with CoCl_2 for additional 24 hours blocked the activation of caspase3 substantially. CoCl_2 at 500 μM caused stabilization of HIF1 α in the nuclear extracts and this stabilization started as early as three hours after treatment (Figure 3B). This confirmed induction of hypoxia. PQ1 alone did not cause activation or stabilization of caspase3 or HIF1 α respectively. PQ1, CoCl_2 or a combination of both did not cause any change in the Cx43 gap junction protein levels or phosphorylation of Cx43 at residue ser368. Activation of Caspase3 and stabilization of HIF1 α indicates hypoxia induced apoptosis in CoCl_2 treated cells. Pre-treatment with PQ1 was able to prevent the activation of Caspase3 by CoCl_2 .

To confirm apoptosis, Annexin V-FITC / PI staining of cells was done. The early apoptotic stage is characterized by the cell membrane exposure of phosphatidylserine normally restricted to the inner cell membrane, which is recognized by annexin V-FITC. The later phase of apoptosis is assessed by measuring the DNA labeling with the PI, an indicator of the cell membrane permeabilization. Once again, CoCl_2 at 500 μM for 24 hours was found to cause significant apoptosis (Figure 4A & B). Pre-treatment of R28 cells with 10 μM PQ1 for 40 min followed by incubation with CoCl_2 at 500 μM protected the cells significantly from undergoing apoptosis (Figure 4). Treatment of cells only with 10 μM PQ1 did not cause any damage to the cells even after 36 hours (Figure 4C).

These data suggest that inhibition of gap junctions by PQ1 protects cells from CoCl_2 -induced ischemic apoptosis. This class of drugs could provide a novel method to prevent the damage which is known to occur during ischemic insult. There are very few known gap junction inhibitors. PQ1 is shown herein to be an excellent gap junction inhibitor which is not toxic and prevents retinal cell apoptosis.

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Fig. 1. Molecular formulas of PQs and computational docking

(A) PQ analogs were synthesized via a modification of the reported protocol [25–27]. The succinic acid salt of PQ1 was prepared to provide water-soluble material for biological evaluation. HCl salts of PQ2, 3, and 4 were also made and they are soluble in water. Succinic acid alone does not show bioactivities. (B) Computational docking of gap junction hemichannel (the hexameric connexon is marked in blue) and PQ1 (marked in red). (C) Interactions between PQ1 (marked in blue) and two helical bundles of a connexin protein (marked in green and orange).

Fig. 2. PQ1 inhibition of gap junction dye transfer activity in retinal neurosensory R28 cells in culture

R28 cells were grown in 6-well plates with coverslips. When cells reached 90 % confluency, gap junction dye transfer activity was performed as described in Methods Section. 2A shows the transfer of Lucifer yellow dye in the control and PQ1 treated cells. 2B is the bar graph of percentage dye transfer in R28 cells after treatment with the different PQs and Mefloquine (MQ). Application of PQ1 significantly (*) inhibited gap junction activity.

A **B****Fig. 3. Protection of PQ1 from CoCl₂-induced hypoxia in R28 cells**

(A) About 70 % confluent retinal R28 cells were treated with 100, 200 and 500 μM CoCl₂ for 24 hrs with or without the pre-treatment of PQ1 (10 μM, 40 min) in a cell culture chamber (5 % CO₂, room air, 37° C). Caspase3 activation was determined by Western blotting in whole cell homogenates (WCH). (B) HIF1α stabilization was measured in the nuclear extracts (NE) after treatment with 500 μM CoCl₂ at different time intervals with or without the pre-treatment of PQ1. CoCl₂ treatment stabilized HIF1α levels in the NE as early as after three hours; PQ1 alone did not have any effect on HIF1α stabilization even after 24 hours. Levels of Cx43 and phosphoCx43-Ser368 are measured in WCH. β-actin is used as a loading control.

Fig. 4. Apoptosis assay using the Annexin V-FITC Kit

(A) Representative flow cytometer images of R28 cells with different treatments of PQ1 and/or CoCl₂. The y-axis quantifies the number of cells stained with propidium iodide and the x axis quantifies number of cells stained with Annexin V-FITC. (B) and (C) Histogram of % apoptotic cells after treatment with CoCl₂ and PQ1. The percentage of apoptotic cells represents cells that are Annexin V-FITC positive and both propidium iodide and Annexin V-FITC positive after different time periods.