An Artificial Copper Complex Incorporating a Cell-Penetrating Peptide Inhibits Nuclear Factor- κ B (NF- κ B) Activation

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Nuclear factor- κ B (NF- κ B) is an inducible transcription factor activated by a variety of cytokines, and promotes the transcription of genes involved in cancer, inflammation, autoimmune disease, and viral infection, among others. Because of its involvement in numerous disease processes, considerable research has focused on NF- κ B as a potential drug target. We previously reported that cupric ion (Cu^{2+}) blocks NF- κ B activation. However, Cu^{2+} is unsuitable for drug applications. The copper complex of an artificial peptide HPH-Pep (HPH-Pep- Cu^{2+}) was a promising alternative, but it did not easily cross the cell membrane. We report the development of a NF- κ B inhibiting Cu^{2+} complex with improved cell-penetrating activity arising from the coupling of a Tat peptide to HPH-Pep- Cu^{2+} .

Key words nuclear factor- κ B (NF- κ B); copper complex; cell-penetrating peptide; Tat

Nuclear factor- κ B (NF- κ B) is an inducible transcription factor activated by extracellular stimuli from a diverse array of cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), and the CD40 ligand.^{1,2)} While both TNF- α and IL-1 activate NF- κ B *via* a canonical pathway, the CD40 ligand activates NF- κ B also by way of a noncanonical pathway. In the canonical pathway, NF- κ B (p50/RelA heterodimer) is sequestered in the cytoplasm in an inactive form associated with the inhibitory protein I κ B. Signaling by cytokines such as TNF- α and IL-1 induces I κ B kinase (IKK) β -dependent and NF- κ B essential modulator (NEMO)-dependent phosphorylation of I κ B, which leads to ubiquitin-dependent degradation of I κ B and subsequent nuclear translocation of NF- κ B. In the nucleus, NF- κ B binds to the κ B sequence found in DNA, thereby promoting the transcription of genes involved in a number of cellular processes, including transformation, survival, proliferation, invasion, angiogenesis, metastasis, inflammation, and virus replication.

Due to its important role in many cellular functions, considerable research has focused on NF- κ B as a potential target of drugs such as anticancer and anti-inflammatory ones,^{3–5)} some of which have already received clinical approval.⁴⁾ However, since NF- κ B functions pleiotropically in normal cells, the specificity of drugs targeting NF- κ B must be carefully tailored in order to avoid side-effects.³⁾ One way to

overcome problems associated with specificity is to control the means of drug delivery.⁵⁾

We previously reported that cupric ion (Cu^{2+}) blocks NF- κB activation by inhibiting the signal-induced phosphorylation of I κB .⁶⁾ Intake of excess free copper, however, causes copper poisoning.⁷⁾ Furthermore, the delivery of free metal ions in the body cannot be controlled. Since organic Cu^{2+} complexes can be chemically modified to control their delivery, they are considered promising alternatives to the use of free copper for blocking NF- κB activation. Here, we report the development of a new NF- κB inhibiting Cu^{2+} complex that incorporates a cell-penetrating peptide.

Results

We previously reported the synthesis of the artificial peptide HPH-Pep, which is comprised of a pyridine and two histidine units (Fig. 1).⁸⁾ A Cu²⁺ complex of HPH-Pep (HPH-Pep-Cu²⁺) demonstrated superoxide-scavenging activity. We initially compared the effect of HPH-Pep-Cu²⁺ and Cu²⁺ on IL-1 α -induced activation of NF- κ B in HeLa S3 cells. Cells were co-transfected with 3 κ B-tk-luc (which has a triple κ B sequence that binds to NF- κ B joined to a downstream firefly luciferase gene) and pRL-Luc (which carries a β -actin promoter joined to a downstream renilla luciferase gene, and which served as an internal control). A solution (final

Fig. 1. Structures of the Artificial Ligands Used in This Study

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500 μM) of Cu^{2+} , HPH-Pep, or HPH-Pep- Cu^{2+} was added to the medium at 20 h post-transfection and the cells were incubated for 1 h. Next, IL-1 α (final 10 ng/ml) was added to the medium and the cells were incubated for an additional 3 h. Cell lysates were then prepared, and a dual-luciferase assay was performed. The firefly luciferase activity was normalized to the renilla luciferase activity.

The results of this experiment are shown in Fig. 2A. While the luciferase activity was barely detectable in the absence of IL-1 α , it increased dramatically in the presence of IL-1 α , confirming that NF- κB is activated by IL-1 α in this experimental system. When 500 μM Cu^{2+} was present in addition to IL-1 α , the luciferase activity decreased to about half that detected with IL-1 α alone, as has been previously observed in Jurkat cells.⁶⁾ Conversely, neither the HPH-Pep ligand or HPH-Pep- Cu^{2+} complex had a clear inhibitory effect on luciferase activity.

Based on the above results, we hypothesized that HPH-Pep- Cu^{2+} does not penetrate the cell membrane, while Cu^{2+} does. HeLa S3 cells were transfected and treated with either a Cu^{2+} or HPH-Pep- Cu^{2+} solution (final 500 μM) and IL-1 α , washed 10 times with phosphate buffered saline (PBS), lysed, and then the amount of intracellular copper was determined using atomic absorption spectrometry. The copper content of cells treated with HPH-Pep- Cu^{2+} was less than half that of cells treated with Cu^{2+} alone, indicating that the membrane-permeability of HPH-Pep- Cu^{2+} is lower than that of Cu^{2+} (Fig. 2B). This could be one reason why HPH-Pep- Cu^{2+} does not inhibit NF- κB activation. We also examined the effect of reducing the number of washes to five and found that five washes were sufficient to remove extraneous copper prior to cell lysis (data not shown).

We then attempted to improve the cell-penetrating ability of HPH-Pep- Cu^{2+} by incorporating the cell-penetrating peptide Tat⁹⁻¹¹⁾ (RKKRRQRRR) (HPH-Pep-Tat; Fig. 1). Synthesis of HPH-Pep-Tat was carried out as shown in Chart 1. First, Pmc/Mtr/Trt/Boc-protected Tat peptide was synthesized using Fmoc solid-phase peptide synthesis on Wang resin.¹²⁾ Next, Trt/Fmoc-protected L-histidine was coupled to the N-terminus of the peptide using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/diisopropylethylamine (DIPEA), followed by Fmoc deprotection with piperidine treatment. A crude sodium salt of histamine-pyridine unit **1**, which was obtained by alkaline hydrolysis of the corresponding methyl ester,¹³⁾ was added to the peptide-conjugated resin using TBTU/DIPEA. The synthesized peptide was deprotected and cleaved from the resin using trimethylsilyl bromide (TMSBr)/ethanedithiol (EDT)/*m*-cresol/thioanisole/trifluoroacetic acid (TFA). The crude product was washed with ether and purified using reversed phase HPLC to provide HPH-Pep-Tat.

We then examined both the inhibitory activity of the HPH-Pep-Tat Cu^{2+} complex (HPH-Pep-Tat- Cu^{2+}) against NF- κB activation and the intracellular copper content of cells treated with HPH-Pep-Tat- Cu^{2+} using the same methods described above. The results are shown in Figs. 3A and B. In the presence of 500 μM HPH-Pep-Tat- Cu^{2+} , the luciferase activity decreased by approximately half, as was observed in the presence of Cu^{2+} (Fig. 3A). However, 50 μM HPH-Pep-Tat- Cu^{2+} had no effect on luciferase activity (data not shown). Copper uptake by cells treated with HPH-Pep-Tat- Cu^{2+} was essentially the same as that of cells treated with free Cu^{2+} (Fig. 3B). These results confirmed that we had developed a new Cu^{2+} complex that inhibits NF- κB activation.

Fig. 2. Biological Activity of HPH-Pep- Cu^{2+}

(A) NF- κB inhibitory activity. HeLa S3 cells were co-transfected with 1.7 μg of 3 κB -tk-luc and 1.7 μg of pRL-Luc. The designated compound (500 μM) was added to the medium at 20 h post-transfection and the cells were incubated for 1 h. Then IL-1 α (10 ng/ml) was added to the medium and the cells were incubated for an additional 3 h. Cells were then lysed and a dual-luciferase assay was performed. Firefly luciferase activity was normalized to renilla luciferase activity in order to calculate the relative luciferase activity. (B) Intracellular copper content. HeLa S3 cells were transfected and treated with the designated compound and IL-1 α as described in (A). The cells were then washed 10 times with PBS, lysed, and analyzed using atomic absorption spectrometry. The copper content of untreated control cells was subtracted from each value.

Synthesis of HPH-Pep-Tat. (i) Fmoc solid-phase peptide synthesis. (ii) Treatment with TBTU/DIPEA followed by piperidine. (iii) Treatment with TBTU/DIPEA. (iv) Treatment with TBSBr/EDT/*m*-cresol/thioanisol/TFA.

Chart 1

Fig. 3. Biological Activity of HPH-Pep-Tat-Cu²⁺

(A) NF- κ B inhibitory activity (determined as described in Fig. 2A). (B) Intracellular copper content (determined as described in Fig. 2B).

Discussion

In this study, we found that although the artificial Cu²⁺ complex HPH-Pep-Cu²⁺ has low ability to penetrate the cell membrane, introduction of the cell-penetrating Tat peptide into the structure of the molecule increases the cellular uptake of the complex. Both HPH-Pep-Tat-Cu²⁺ and free Cu²⁺ had nearly identical capacity to penetrate the cell membrane; however, the mechanism of penetration employed by each is considered to be different. While Cu²⁺ enters a cell *via* copper transporters,¹⁴⁾ compounds incorporating cell-penetrating

peptides cross the cell membrane by endocytosis and/or direct penetration.^{9,10)} Thus, by incorporating a cell-penetrating peptide into a Cu²⁺ complex, we succeeded in introducing copper into a cell presumably without involving copper transporters.

We also found that HPH-Pep-Tat-Cu²⁺ inhibits NF- κ B activation in a manner similar to free Cu²⁺. When we first reported this function of Cu²⁺ in 1995,⁶⁾ it was widely believed that reactive oxygen species (ROS) regulate the NF- κ B activation signaling pathway. We therefore hypothesized that the

inhibitory activity associated with Cu^{2+} is related to ROS.⁶⁾ However, in 2003 it was reported that ROS do not mediate NF- κ B activation.¹⁵⁾ Since the precise role of ROS in NF- κ B activation is still a matter of contention,^{3,16)} we cannot at this point speculate as to the mechanism whereby Cu^{2+} and HPH-Pep-Tat- Cu^{2+} inhibit NF- κ B activation.

Though the membrane permeability and the inhibitory activity of NF- κ B activation of HPH-Pep-Tat- Cu^{2+} and free Cu^{2+} are comparable, free copper is soon captured, *in vivo*, by various copper-transporting/storage proteins (e.g., ceruloplasmin, albumin, or metallothionein), preventing copper from occurring as toxic free ionic metal.¹⁷⁾ Thus, HPH-Pep-Tat- Cu^{2+} could be an alternative to free Cu^{2+} . In fact, recent research suggests that organic complexes of copper are useful as proteasome inhibitors, anticancer agents, and in the treatment of Menkes disease.¹⁴⁾ Further chemical modification of the HPH-Pep-Tat ligand would lead to the development of not only a simple NF- κ B inhibitor, but also a variety of biologically active copper complexes.

Experimental

General Procedures Pertaining to Synthesis Melting points were determined using a Yanaco Micro Melting Point Apparatus (Yanaco, Tokyo, Japan). Mass spectra matrix assisted laser desorption/ionization time-of-flight (MALDI TOF-MS) were recorded using a Bruker REFLEX MALDI TOF-MS (Bruker, Madison, WI, U.S.A.). High-resolution mass spectra (FAB-HR-MS) were recorded using a JEOL JMS-DX303HF mass spectrometer (JEOL, Tokyo, Japan).

HPH-Pep-Tat To prepare HPH-Pep-Tat, 6-[2-(1*H*-imidazol-4-yl)ethyl-carbamoyl]pyridine-2-carboxylic acid methyl ester¹³⁾ was dissolved in methanol and a solution of 1 *N* NaOH in methanol was added. The resulting reaction mixture was heated under reflux for 1 d. The methanol was removed under vacuum, and the resulting residue was washed with dichloromethane to provide crude product **1** (Chart 1). The peptide was synthesized by Fmoc solid-phase peptide synthesis using an EYELA Personal Organic Synthesizer CCS-600 (Tokyo Rikakikai, Tokyo, Japan). Briefly, a side chain protected peptide (HRKKRRQRRR) conjugated to resin (HRKKRRQRRR-resin) was synthesized from Fmoc-L-Arg(Pmc)-Wang resin, Fmoc-L-Arg(Mtr)-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-Lys(Boc)-OH, and Fmoc-L-His(Trt)-OH. The conjugation and deprotection reactions were performed with TBTU/DIPEA and piperidine, respectively. Next, product **1** was conjugated to the protected peptide (HRKKRRQRRR-resin) using TBTU/DIPEA, and then the final steps of Pmc/Mtr/Trt/Boc deprotection and cleavage of the synthesized peptide from the resin were performed using TMSBr/EDT/*m*-cresol/thioanisole/TFA. The crude product was washed with ether and purified using reversed phase HPLC [column: STR ODS-II, 150×4.6 mm, Shinwa Chemical Industries, Kyoto, Japan; eluent: 0.1% TFA in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (6—17% gradient over 30 min); flow rate: 1.0 ml/min] to provide HPH-Pep-Tat. Mp 120.0—120.5 °C. MS (MALDI TOF) $M_{\text{r,calc}}=1718$, $M_{\text{r,meas}}=1719$ ($M+H^+$). FAB-HR-MS Calcd m/z for $\text{C}_{71}\text{H}_{124}\text{N}_{37}\text{O}_{14}$ 1719.0129. Found: 1719.0287 ($M+H^+$).

Cu^{2+} and Its Complexes A 100X stock Cu^{2+} solution was prepared by dissolving CuSO_4 in water. An aliquot of the CuSO_4 stock solution was mixed with an equal volume of 100X HPH-Pep or HPH-Pep-Tat in dimethyl sulfoxide (DMSO) and vortexed to prepare the HPH-Pep and HPH-Pep-Tat complexes.

Plasmids and Cytokines The 3 κ B-tk-luc vector (provided by Dr. Shigeki Miyamoto, The University of Wisconsin, U.S.A.) has three κ B sequences joined to a downstream firefly luciferase gene. The pRL-Luc vector has a β -actin promoter joined to a downstream renilla luciferase gene. Cells were stimulated with the cytokine IL-1 α (PeproTech, Rocky Hill, NJ, U.S.A.).

Cell Culture, Transfection, and Luciferase Assay HeLa S3¹⁸⁾ cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). The HeLa S3 cells were transfected using the calcium-phosphate co-precipitation method. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, U.S.A.).

Measurement of Intracellular Copper¹⁹⁾ Cells were washed 10 times with PBS and suspended in 0.3 ml of 15 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (in 0.9% NaCl, pH 7.3). The cell suspension was mixed with 0.9 ml of 0.1 *N* HNO_3 and incubated at 60 °C for 4 h. The copper content of the resulting cell lysate was determined using a PerkinElmer Simaa 6000 atomic absorption spectrometer (PerkinElmer, Waltham, MA, U.S.A.).

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