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Thujaplicin–copper chelates inhibit replication of human influenza viruses

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Abstract

The effects of α-, β- and γ-thujaplicins and six of their metal chelates on human influenza virus-induced apoptosis in Madin–Darby canine kidney (MDCK) cells were examined by DNA fragmentation and flow cytometry. Among the compounds tested, thujaplicin–copper chelates inhibited apoptosis induced in the infected MDCK cells with influenza A/PR/8/34(H1N1), A/Shingapol/1/57(H2N2), A/Aichi/2/68(H3N2) and B/Lee/40 viruses, at concentrations of more than 5 μM. These results indicate that the copper chelates inhibit influenza virus-induced apoptosis and that the inhibitory effects may be independent of influenza virus subtype or types. Furthermore, the copper chelates also inhibited the release of the viruses from the infected MDCK cells during apoptosis. The anti-apoptotic effects of the copper chelates may occur 2–4 h postinfection, suggesting that the copper chelates affect MDCK cells directly in the early stage of influenza virus-induced apoptosis. In this study, we demonstrated that thujaplicin–copper chelates inhibit influenza virus-induced apoptosis of MDCK cells and also inhibit virus replication and release from the infected cells.

Keywords: Antiviral effect; Apoptosis; Influenza virus; Thujaplicin–metal chelates

1. Introduction

Thujaplicins, including β-thujaplicin (2-hydroxy-4-isopropyl-2,4,6-cyclohepentatrieneone), α-thujaplicin (2-hydroxy-3-isopropyl-2,4,6-cycloheptatrienone) and γ-thujaplicin (2-hydroxy-5-

Abbreviations: BSA, bovine serum albumin; ED_{50}, 50% effective dose; EMEM, Eagle's minimal essential medium; FCS, fetal calf serum; HBSS, Hank's buffered saline solution; LDH, lactate dehydrogenase; MDCK, Madin–Darby canine kidney; PBS, phosphate buffered saline; PI, propidium iodide.

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isopropyl-2,4,6-cycloheptatrienone), are tropolone-related compounds found in the heartwood of several cupressaceous plants such as western red cedar (*Thuja plicata*), eastern white cedar (*Thuja occidentalis*) and hinoki cypress (*Chamaecyprais obtusa*) (Nozoe, 1936, Edman and Gripenberg, 1948). Thujaplicins have been reported to possess antibacterial and antifungal activities (Anderson and Gripenberg, 1948, Edman and Gripenberg, 1948, Pennerfelt, 1948, Pauson, 1955, Trust and Coombs, 1973, Akers et al., 1980) and also to exhibit antitumor activity (Yamato et al., 1986, Inomori et al., 1993). Thujaplicins have a β-diketone moiety in the structures and form chelate in the presence of various metal ions, such as copper ions and zinc ions (MacLean and Gardner, 1956, Endo et al., 1988). However, anti-influenza virus effects of thujaplicins and their metal chelates remain to be examined.

Recent studies have demonstrated that cell death induced by influenza virus infection occurs by apoptosis (Takizawa et al., 1993, Hinshaw et al., 1994, Takizawa et al., 1995). This has been suggested to a host defence mechanisms, but virus replication and release from infected cells occur during influenza virus-induced apoptosis (Takizawa et al., 1993). Therefore, reagents capable of efficiently inhibiting virus-induced apoptosis could become candidate anti-influenza virus drugs that could prevent infection of host cells with influenza viruses during apoptosis. In addition, such reagents could be useful for analyzing the mechanism of virus-induced apoptosis. In this report, we focus on the apoptosis induced in human influenza virus-infected MDCK cells, and examine the effects of thujaplicins and their metal chelates on human influenza virus-induced apoptosis.

2. Materials and methods

2.1. Reagents

Natural β-thujaplicin (2-hydroxy-4-isopropyl-2,4,6-cyclohepentatrieneone), synthetic α-thujaplicin (2-hydroxy-3-isopropyl-2,4,6-cycloheptatrienone) and synthetic γ-thujaplicin (2-hydroxy-5-isopropyl-2,4,6-cycloheptatrienone) were kindly provided by Takasago (Tokyo, Japan). All thujaplicin–metal chelates, such as thujaplicin–zinc chelate, thujaplicin–copper chelate, thujaplicin–ferrous chelate, thujaplicin–ferric chelate, thujaplicin–magnesium chelate and thujaplicin–manganese chelate, were prepared as follows. Briefly, thujaplicin was dissolved in methanol at a concentration of 150 mM, and then each metal salt was added at a final concentration of 80 mM. After mixing for 5 h followed by paper filtration, the mixtures were washed three times with Mili Q water to exclude free metal ions, and dried under vacuum. Thujaplicins and the metal chelates were dissolved in dimethyl sulfoxide (DMSO; Sigma) at 40 mM, and stocked in the dark at -40°C until use. The final concentrations (0.0025–0.05%) of DMSO did not influence the growth or viability of the MDCK cells.

2.2. Influenza viruses and cell culture

Influenza A virus isolates [A/PR/8/34(H1N1), A/Shingapol/1/57(H2N2), and AtAichi/2/68 (H3N2)] and influenza B virus (B/Lee/40) were grown in the allantoic cavity of 10-day-old embryonic chicken eggs incubated at 34°C, and purified by sucrose density gradient centrifugation as described previously (Suzuki et al., 1980). Viral hemagglutination units were determined at 4°C in microtiter plates as described previously (Suzuki et al., 1983). Madin–Darby canine kidney (MDCK) cells were maintained in Eagle's minimal essential medium (EMEM) (pH 7.2) containing 60 mg/l of kanamycin sulfate and 10% (v/v) fetal calf serum (FCS) at 37°C in a 5% CO_2 atmosphere. Virus plaque assay was performed as described previously (Takizawa et al., 1993). In brief, confluent monolayers of MDCK cells were infected with virus solution for 1 h at room temperature, overlaid with 0.6% agarose containing 2.5 mg/ml of trypsin, and then incubated for 3 days at 34°C. Cells were stained with 1% crystal violet in 20% ethanol.

2.3. Infection of viruses to MDCK cells

Subconfluent monolayers of MDCK cells were infected with each virus at a multiplicity of infection (m.o.i.) of 20 for 60 min at 4°C as described previously (Takizawa et al., 1993). The infected cells were washed twice with phosphate buffered saline (PBS) and then incubated in EMEM containing 0.5% FCS, in the presence or absence of samples, at 34°C for 16 h. The infected and mock-infected cell cultures were examined under a light microscope for the progression of virus-induced cytopathic effects and were then further examined by DNA fragmentation and flow cytometric analysis. The cytopathic effects were denoted as follows: –, no morphological changes; ±, slight morphological changes without cell rounding; +, less than 50% of cells rounded; ++, more than 50% of cells rounded and many cells afloat; +++, almost total destruction of monolayers.

2.4. Lactate dehydrogenase (LDH) release assay

The activity of lactate dehydrogenase (LDH) released from MDCK cells for measurement of virus-induced cytolysis of test samples against MDCK cells was determined according to a modification of the colorimetric assay described previously (Suzuki et al., 1996). The assays were carried out in triplicate.

2.5. DNA preparation and gel electrophoresis

Electrophoresis of total DNA was performed as described previously with slight modifications (Takeda et al., 1993, McCloskey et al., 1994). Briefly, after the monolayer cells infected with each virus were rinsed twice with PBS, PBS containing 0.05 mM EDTA was added to the dishes and then incubated for 15 min at 37°C. After incubation, the cells were collected and further washed twice with PBS. Harvested cells, which were simultaneously analyzed by flow cytometry, were centrifuged, and then 2×10^6 cells were resuspended in 20 μl of lysis buffer (10 mM EDTA, 50 mM Tris–HCl pH 8.0, 0.5% (w/v) sodium dodecyl sulfate, 0.5 mg/ml proteinase K) followed by incubation for 1 h at 50°C. After the incubation and phenol–chloroform extraction, RNase A was added to a final concentration of 0.5 mg/ml, and the samples were subsequently incubated at 50°C for 1 h. After incubation, all treated samples were electrophoresed through a 1.6% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and photographed. The degree of DNA fragmentation was expressed as follows: –, no fragmentation of cellular DNA; ±, fragmentation of cellular DNA the same as that occurring spontaneously in cultured cells; +, slightly more DNA fragmentation than that occurring spontaneously; ++, high level of DNA fragmentation, but less than that in infected cells at m.o.i. of 10; +++, same level of DNA fragmentation as that in infected cells at m.o.i. of 20.

2.6. Flow cytometric analysis

Flow cytometric analysis using propidium iodide (PI) was performed as described previously (Takeda et al., 1993). Briefly, 2×10^6 cells were harvested as described above, and then fixed in 70% ethanol for 1 h at 4°C. The cells were washed and resuspended in 0.5 ml of Hank's buffered saline solution (HBSS) to which 0.5 ml of RNase A solution (1 mg/ml in HBSS) was added, followed by 1 ml of PI solution (100 mg/ml in HBSS). Following gentle mixing, samples were maintained at 4°C in the dark overnight. PI fluorescence of each cell was assessed by analysis on an Epics Elite flow cytometer (Coulter, Hialeah, FL) for the presence of a sub-G_0 (hypodiploid or A_0) peak.

3. Results

3.1. Cytotoxic effects of thajaplicins and the thajaplicin–metal chelates on MDCK cells

In a preliminary experiment, we examined the cytotoxic effects of α-, β- and γ-thujaplicins and

six of their metal chelates on MDCK cells by LDH release assay. No cytotoxic effects of the thujaplicins and the metal chelates were observed at concentrations below 60 μM and 20 μM, respectively. On the basis of the results, all samples were used at concentrations below 20 μM in subsequent experiments. Cell viability was also determined after treatment with each sample at 20 μM for 16 h, and was more than 92%.

3.2. Determination of m.o.i. for virus-induced apoptosis

To determine the optimal concentrations of human influenza virus solutions that induce apoptosis of MDCK cells, the cells were infected with the human influenza viruses A/Aichi/2/68(H3N2), A/PR/8/34(H1N1), A/Shingapol/1/57(H2N2) and B/Lee/40 at m.o.i. of 20, 10, 5 and 2, respectively. The viral infection at m.o.i. of more than 10 significantly induced apoptosis in MDCK cells. Furthermore, we examined the effects of zinc ions (Zn^{2+}) on apoptosis in the influenza virus-infected MDCK cells, as Zn^{2+} is a well-defined inhibitor of the endonucleases responsible for the DNA degradation observed in apoptosis. The addition of 0.1 mM Zn^{2+} significantly inhibited apoptosis induced by the viruses at m.o.i. below 10 but not at 20 m.o.i. (data not shown). On the basis of these results, to examine the inhibitory effects of test samples on virus-induced apoptosis the subsequent experiments were performed at m.o.i. of 20.

3.3. Effects of thajaplicins and their metal chelates on apoptosis induced in MDCK cells infected with human influenza A/Aichi/2/68(H3N2)

We examined the effects of thujaplicins and their metal chelates on apoptosis induced in MDCK cells infected with human influenza virus A/Aichi/2/68(H3N2) at concentrations of 10 μM, assessed by cell viability, cell morphological change, DNA fragmentation assay and flow cytometric analysis. A typical profile for the effect of β-thujaplicin–copper chelate on virus-induced apoptosis in MDCK cells assessed by DNA fragmentation assay and flow cytometric analysis is shown in Fig. 1. Thujaplicin–copper chelates showed the most effective inhibition against the virus-induced apoptosis; γ-thujaplicin, although less effective than the copper chelates, also showed inhibitory effects (Table 1). In addition, other thujaplicin isomers and metal chelates such as thujaplicin–ferrous, thujaplicin–ferric, thujaplicin–magnesium and thujaplicin–manganese chelates showed no inhibition of virus-induced apoptosis (data not shown). The minimum concentration of each of the copper chelates required for the inhibition of apoptosis was approximately 5 μM, indicating that thujaplicin–copper chelates are potent inhibitors of virus-induced apoptosis. On the other hand, β-thujaplicin, the copper chelate and the zinc chelate did not influence apoptosis occurring in the mock-infected MDCK cells (Table 1). Similar results were obtained also with other isomers and their metal chelates. These results indicate that the compounds might not inhibit apoptosis occurring in the uninfected cells.

3.4. Inhibitory effects of thujaplicin–copper chelates on apoptosis induced by influenza viruses of different antigenic types (A, B) and subtypes (H1–H3; H1N2)

We examined whether thujaplicin–copper chelates could inhibit apoptosis induced by influenza viruses of different antigenic types and subtypes, i.e. influenza A/PR/8/34(H1N1), A/Shingapol/1/57(H2N2) and B/Lee/40 viruses. As shown in Table 2, the compound inhibited apoptosis induced by infection with these viruses as well as human influenza virus A/Aichi/2/68(H3N2). In addition, other thujaplicin–copper chelates, i.e. γ- and α-thujaplicin–copper chelates, also exhibited similar inhibitory effects on the apoptosis induced by these viruses, but other metal chelates, such as the ferrous chelates, the ferric chelates, the magnesium chelates and the manganese chelates, did not (data not shown). These results indicate that the inhibitory effects of thujaplicin–copper chelates on influenza virus-induced apoptosis are independent of subtype and type of influenza virus.

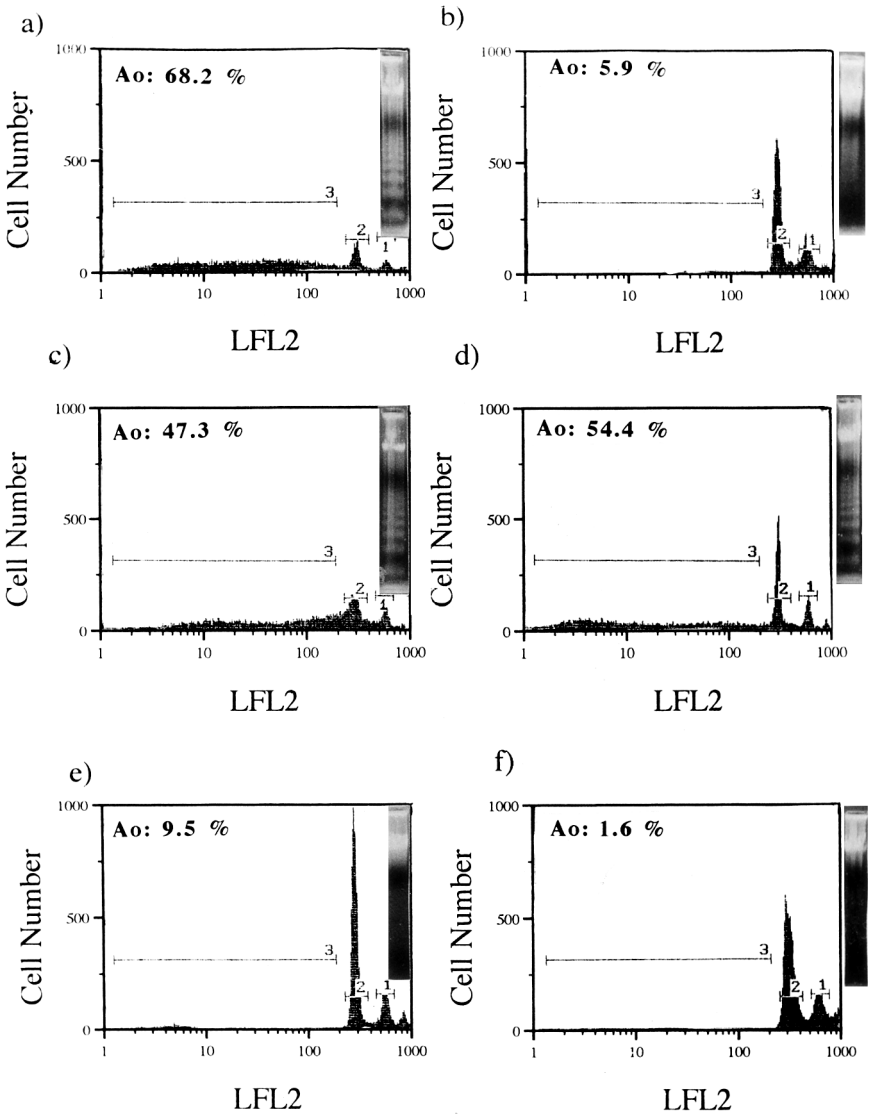

Fig. 1. The effect of β-thujaplicin and the copper chelate on apoptosis induced in MDCK cells infected with influenza A/Aichi/2/68(H3N2) virus by gel electrophoresis and flow cytometric analysis. MDCK cells (1×10^6 cells) were infected for 60 min with influenza A/Aichi/2/68(H3N2) virus at m.o.i. of 20 (a, c), or were mock-infected (b), and further incubated for 40 h (a) or 16 h (b, c) in culture medium alone. To examine the effects of β-thujaplicin alone and β-thujaplicin–copper chelate, the MDCK cells infected with the virus at m.o.i. of 20 (d, f) or without the virus (e) were incubated for 16 h in the medium containing 10 μM of β-thujaplicin alone (d) or 10 μM of β-thujaplicin–copper chelate (e, f). PI-stained cells and DNAs were prepared as described in Section 2, and identical samples were simultaneously analyzed by flow cytometry and gel electrophoresis. DNAs (1×10^6 cells equivalent) were separated by 1.6% agarose gel electrophoresis followed by staining with ethidium bromide. Fluorescence intensities of cells in G_2/M phase (1), G_1 phase (2) and apoptotic phase (3) are shown. Ao, percentage of apoptotic to total cells.

Table 1
Inhibition of human influenza A/Aichi/2/68(H3N2) virus-induced apoptosis by thujaplicins and thujaplicin–metal chelates

Virus		Viability (%)	Morphological changes (c.p.e.)	DNA fragmentation	PI staining (Ao)
–		98.7 ± 1.1	–	±	5.7 ± 1.0
+		68.7 ± 3.4	++	+++	54.7 ± 3.5
–	β-Thujaplicin	96.7 ± 2.1	–	±	6.0 ± 1.4
–	β-Thujaplicin	98.7 ± 1.0	–	±	4.5 ± 0.5
	–copper				
–	β-Thujaplicin–zinc	97.9 ± 3.5	±	±	5.0 ± 2.1
–	Cu^{2+}	97.8 ± 2.5	–	±	4.0 ± 1.5
–	Zn^{2+}	98.8 ± 0.8	–	±	3.0 ± 2.5
+	β-Thujaplicin	76.7 ± 3.1	+	++	52.5 ± 2.3
+	β-Thujaplicin	100	–	–	1.3 ± 0.1 *
	–copper				
+	β-Thujaplicin–zinc	82.9 ± 3.5	+	++	49.5 ± 1.4
+	α-Thujaplicin	75.0 ± 6.1	+	++	51.5 ± 4.5
+	α-Thujaplicin–copper	100	–	–	1.2 ± 0.1 *
+	α-Thujaplicin–zinc	83.3 ± 1.1	–	++	46.4 ± 2.2
+	γ-Thuiaplicin	90.0 ± 3.1	+	+	36.3 ± 1.0 **
+	γ-Thuiaplicin–copper	100	–	–	1.0 ± 0.2 *
+	γ-Thujaplicin–zinc	75.9 ± 1.8	+	+	50.1 ± 2.0
+	Cu^{2+}	84.7 ± 2.5	+	+++	53.5 ± 3.5
+	Zn^{2+}	75.7 ± 0.8	+	+++	47.5 ± 1.2

MDCK cells were infected with influenza A/Aichi/2/68(H3N2) virus at m.o.i. of 20 for 60 min. After the infection, the cells were washed, and then incubated with 10 μM of each sample for 16 h. After the incubation, apoptosis induced in the infected and uninfected MDCK cells was assessed by cell viability, morphological change, DNA fragmentation and flow cytometry as described in Section 2.
* $P<0.01$.
** $P<0.05$.

3.5. Synergistic effects of thujaplicin and copper ions on apoptosis induced in MDCK cells infected with human influenza viruses

To examine the synergistic effect of thujaplicin and copper ions on virus-induced apoptosis, MDCK cells were infected with human influenza A/Aichi/2/68(H3N2), A/PR/8/34(H1N1), A/Shingapol/1/57(H2N2) and B/Lee/40 viruses; 1–20 μM copper sulfate ($CuS0_4$) solution was then added to culture media containing 10 μM of β-thujaplicin. As shown in Fig. 2, the virus-induced apoptosis effect decreased with increasing concentrations of copper ions and was completely inhibited at about 5 μM. Other thujaplicin isomers, such as α-thujaplicin and γ-thujaplicin, also showed the anti-apoptotic effect at the same molar ratio (data not shown). These results indicate that the copper chelate was formed by chelation of thujaplicin with copper ions in culture medium and that the chelate itself might exhibit anti-apoptotic effects on the virus-infected MDCK cells.

3.6. Effect of time of addition of the copper chelates on virus-induced apoptosis

We examined the effect of the time of β-thujaplicin–copper chelate addition on apoptosis induced with influenza A/Aichi/2/68(H3N2), A/PR/8/34(H1N1), A/Shingapol/1/57(H2N2) and B/Lee/40 viruses. Treatment with β-thujaplicin–copper chelate at or before 2 h postinfection efficiently blocked the virus-induced apoptosis, whereas the treatment at 4 h postinfection or later did not (Fig. 3).

Table 2
Effects of β-thujaplicin–copper chelate and β-thujaplicin–zinc chelate on apoptosis induced in MDCK cells infected with various types of influenza viruses

		Viability (%)	Morphological changes (c.p.e.)	DNA fragmentation	Ao (%)
Mock		92.6 ± 1.0	–	±	7.8 ± 1.0
A/PR/8/34(H1N1)	Medium alone	98.0 ± 0.6	+++	+	23.1 ± 1.0
	β-Thujaplicin	100	–	–	2.5 ± 0.1 *
	–copper				
	β-Thujaplicin–zinc	94.1 ± 2.0	++	±	25.8 ± 1.5
	Cu^{2+}	98.2 ± 1.8	++	+	25.2 ± 0.1
	Zn^{2+}	98.0 ± 3.0	++	+	22.2 ± 0.7
A/Singapol/1/57(H2N2)	Medium alone	88.2 ± 1.1	+++	+	41.5 ± 3.7
	β-Thujaplicin	100	–	–	2.3 ± 0.5 *
	–copper				
	β-Thujaplicin–zinc	81.5 ± 2.0	++	+	29.4 ± 1.1
	Cu^{2+}	88.7 ± 1.3	++	+	34.6 ± 1.1
	Zn^{2+}	88.5 ± 2.5	++	+	37.5 ± 3.0
A/Aichi/2/68(H3N2)	Medium alone	84.4 ± 2.2	+++	++	49.0 ± 1.5
	β-Thujaplicin	100	–	–	2.5 ± 0.5 *
	–copper				
	β-Thujaplicin–zinc	91.0 ± 3.1	++	+	50.6 ± 3.1
	Cu^{2+}	87.2 ± 1.0	++	+	46.7 ± 1.5
	Zn^{2+}	74.7 ± 0.5	++	+	52.9 ± 1.8
B/Lee/40	Medium alone	87.1 ± 1.3	+++	++	37.2 ± 0.3
	β-Thujaplicin	100	–	±	37.0 ± 0.9
	–copper				
	β-Thujaplicin–zinc	89.3 ± 1.1	++		
	Cu^{2+}	87.3 ± 0.8	++	+	38.5 ± 2.9
	Zn^{2+}	89.2 ± 0.9	++	+	33.5 ± 1.0

MDCK cells were infected with each influenza virus at m.o.i. of 20 for 60 min. After the infection, the cells were washed, and then incubated with 10 μM of each sample for 16 h. After the incubation, apoptosis induced in the MDCK cells was assessed by analysis of cell viability, morphological changes, DNA fragmentation and by flow cytometry as described in Section 2.
* $P < 0.001$.

Further, treatment with other thujaplicin–copper chelates, i.e. γ- and α-thujaplicin–copper chelates, also efficiently inhibited the virus-induced apoptosis in the same time-dependent manner (data not shown). These results suggest that the copper chelates might inhibit induction or activation of a host protein(s) that triggers the apoptosis, occurring 2–4 h postinfection or, alternatively, small amounts of viral proteins that had been produced before the addition of the copper chelates at 4 h postinfection, during the early phase of viral growth.

3.7. Effects of thajaplicin–copper chelates on HA titers and plaque titers of viruses generated by influenza virus-induced apoptosis

To determine whether thujaplicin–copper chelates inhibit the release of influenza viruses during the virus-induced apoptosis, we examined

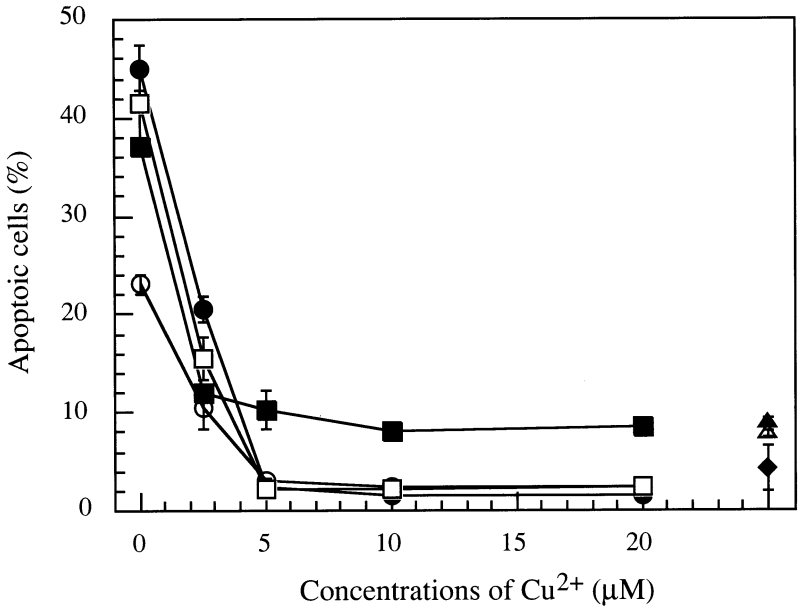

Fig. 2. Synergistic effects of β-thujaplicin and copper ions on apoptosis induced in MDCK cells infected with influenza viruses, assessed by flow cytometric analysis. MDCK cells (1×10^6 cells) were infected with influenza A/Aichi/2/68(H3N2) (●), A/PEV8/34(H1N1) (○), A/Shingapol/1/57(H2N2) (□) and B/Lee/40 (■) viruses for 60 min with m.o.i. of 20. After the incubation, cells were washed with PBS, and then incubated for 16 h in culture medium containing β-thujaplicin (10 μM) and copper sulfate (0–20 μM). The mock-infected MDCK cells were also incubated for 16 h in culture medium containing 10 μM β-thujaplicin (▲), 5 μM copper sulfate (△), or 10 μM β-thujaplicin–copper chelate (◆). After the incubation, the numbers of apoptotic cells were determined by flow cytometric analysis using PI staining. Percentages of apoptotic to total cells are shown. Values are the means of three independent determinations; bars, S.D.

the titers of viral hemagglutination (HA) and the titers of virus in the supernatant of MDCK cells after infection with influenza A/PR/8/34(H1N1) virus at m.o.i. 20 for 16 h in the precence or absence of each thujaplicin–copper chelate (10 μM). As shown in experiment 1 of Table 3, both the HA titers and the virus titers decreased in the supernatant of the infected MDCK cells after incubation with each thujaplicin–copper chelate. Furthermore, we also examined the HA titers and the virus titers in the supernatants after infection of MDCK cells with the influenza virus for 3 h in the presence of each thujaplicin–copper chelate (10 μM). Both titers decreased also in this experment (experiment 2 of Table 3). The same results were obtained for infection with other influenza viruses used in this study (data not shown). The ED_{50} of thujaplicin–copper chelates for the titers of virus in the supernatants after the infection of MDCK cells with each influenza virus for 3 h was about 2 μM.

4. Discussion

Recent reports have demonstrated that influenza virus-induced cell death is apoptotic (Takizawa et al., 1993, Hinshaw et al., 1994, Mori et al., 1995, Takizawa et al., 1995), and virus-induced apoptosis has been suggested to be one of the host defence mechanisms for preventing expansion of viral infection. However, as viral replication and release from the infected cells occur during virus-induced apoptosis (Takizawa et al., 1993), the resultant release of virus particles would result in continuous infection and survival of the virus in infected tissue. Recently Olsen et al. (1996) have suggested that, in the case of influenza virus, apoptosis may be important for optimal viral production. Therefore, reagents capable of selectively blocking virus-induced apoptosis could prevent the subsequent infection of host cells and viral production.

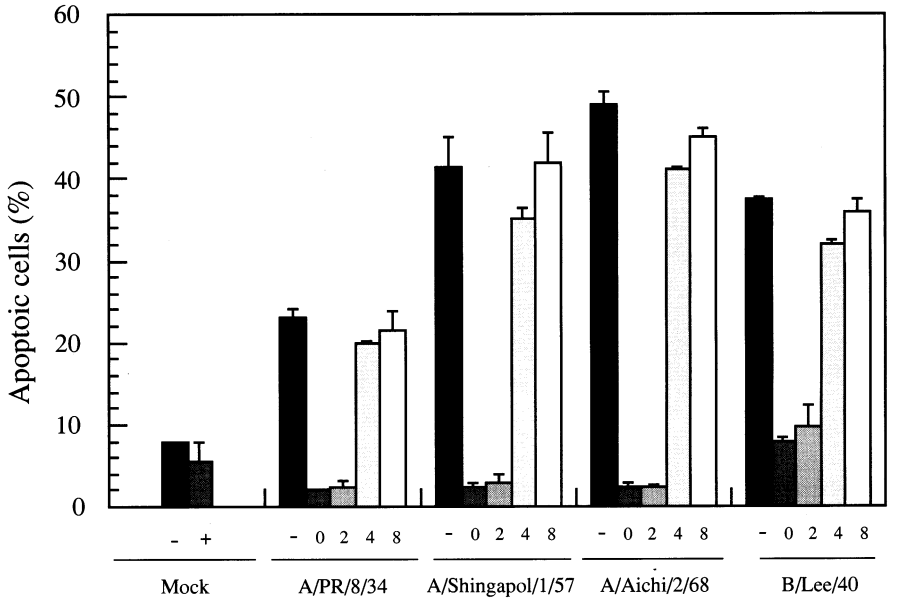

Fig. 3. Effect of time of β-thujaplicin–copper chelate addition on apoptosis induced with influenza viruses. MDCK cells (1×10^6 cells) were mock-infected or infected with influenza A/PR/8/34(H1N1), A/Shingapol/1/57(H2N2) A/Aichi/2/68(H3N2) and B/Lee/40 viruses for 60 min with m.o.i. of 20. For the virus-infected cells, 10 μM β-thujaplicin–copper chelate was added to the medium at 0, 2, 4 or 8 h postinfection. In mock-infected cells, β-thujaplicin–copper chelate was added to the medium at 0 h (+); (–) indicates samples without β-thujaplicin–copper chelate treatment. After the incubation, numbers of apoptotic cells were determined by flow cytometric analysis using PI staining. Percentages of apoptotic to total cells are shown. Values are the means of three independent determinations; bars, S.D.

Thujaplicins have been reported to possess antibactericidal and antifungal activities (Anderson and Gripenberg, 1948, Pennerfelt, 1948, Pauson, 1955, Trust and Coombs, 1973, Akers et al., 1980). Furthermore, β-thujaplicin has been reported to exhibit strong cytopathogenic effects (Okabe et al., 1988), cytotoxic effects of growth in vitro on several tumor cell lines (Inomori et al., 1993) and induction of differentiation in teratocarcinoma F9 cells (Muto et al., 1995). However, the effects of thujaplicins and their metal chelates on influenza virus-induced apoptosis have not yet been examined.

In a preliminanay experment, we examined optimal concentrations of human influenza virus solutions to induce apoptosis on MDCK cells by DNA fragmentation and flow cytometry. Viral infection at m.o.i. of more than 10 significantly induced apoptosis in MDCK cells. These observations indicate that influenza virus infection induces apoptosis in MDCK cells, supporting the results of previous studies (Takizawa et al., 1993, Hinshaw et al., 1994). In addition, the results of flow cytometric analysis suggested that the method is suitable for the quantitative assessment of apoptosis induced in host cells by the viral infection. Zinc ions are a well-defined inhibitor of the endonucleases responsible for the DNA degradation observed in apoptosis (Cohen and Duke, 1984), and the addition of Zn^{2+} (0.1 mM) significantly inhibited apoptosis induced by the viruses at m.o.i. of below 10. Therefore, to screen more potent inhibitors than Zn^{2+}, we examined the inhibitory effects of test samples on the virus-induced apoptosis at m.o.i. of 20.

In this study, we examined the inhibitory effects of α-, β- and γ-thujaplicin and six of their metal chelates on virus-induced apoptosis in MDCK cells. Among the compounds tested, the copper

Table 3
Inhibitiion of the release of influenza viruses during influenza virus-induced apoptosis

	Virus infection	Experiment 1		Experiment 2	
		HA titers	Virus titers (p.f.u./ml)	HA titers	Virus titers (p.f.u./ml)
Medium alone	–	–	0	–	0
Medium alone	+	7	9.0×10^6	7	8.9×10^6
β-Thujaplicin	+	3	4.2×10^5	–	0
–copper					
β-Thujaplicin–zinc	+	7	8.5×10^6	6	8.0×10^6
β-Thujaplicin	+	7	9.0×10^6	6	7.5×10^6
α-Thujaplicin–copper	+	4	1.2×10^6	–	0
α-Thujaplicin–zinc	+	7	9.0×10^6	6	7.5×10^6
α-Thujaplicin	+	7	9.0×10^6	6	7.8×10^6
γ-Thujaplicin–copper	+	3	5.4×10^5	–	0
γ-Thujaplicin–zinc	+	7	9.0×10^6	6	7.5×10^6
γ-Thujaplicin	+	7	9.0×10^6	6	7.6×10^6
Cu^{2+}	+	7	9.0×10^6	6	8.8×10^6
Zn^{2+}	+	7	9.0×10^6	6	8.7×10^6

Experiment 1: MDCK cells were infected with influenza A/PR/8/34(H1N1) virus at m.o.i. of 20, and then the cells were washed. After washing, the cells were incubated for 16 h in the presence or absence of 10 μM of each sample. Experiment 2: MDCK cells were infected with the influenza virus for 3 h in the presence of 10 μM of each sample. After the infection, the cells were washed and then incubated for 16 h in fresh culture medium. After the incubation, the viral hemagglutination (HA) titers and the virus titers (p.f.u., plaque-forming units) in the supernatant of the treated MDCK cells were examined as described in Section 2.
–, HA titers were not detected.

chelates of thujaplicins inhibited influenza A/Aichi/2/68(H3N2) virus-induced apoptosis in MDCK cells at concentrations of more than 5 μM (Table 1). Furthermore, the copper chelates inhibited the apoptosis induced with other influenza viruses, such as A/PR/8/34(H1N1), A/Shingapol/1/57(H2N2) and B/Lee/4O, at concentrations of 10 μM (Table 2), indicating that the inhibitory effects of the copper chelates on influenza virus-induced apoptosis may be independent of influenza virus subtype or types. In a preliminary experiment, we also examined the cytotoxic effects of thujaplicins at concentrations of 5–100 μM on cell growth, morphological changes and viability of MDCK cells. Slight morphological changes were observed at concentrations of more than 5 μM, but cell viability and cell growth of non-infected cells did not decrease signficantly. Inomori et al. (1993) have reported that β-thujaplicin effectively inhibited cell growth of several tumor cells and blastic splenocytes at 1.8–3.6 μM. Compared with these values, similar concentrations are required for inhibition of the virus-induced apoptosis by thujaplicin–copper chelates. Furthermore, we examined whether or not the anti-apoptotic effect of the copper chelates is due to free thujaplicins or the copper chelates themselves in culture medium. The virus-induced apoptotic effect decreased with increasing concentrations of copper ions and the apoptosis was completely inhibited at about 5 μM (Fig. 2). Thujaplicin has been reported to chelate with divalent metal ions at a 2:1 molar ratio (MacLean and Gardner, 1956, Endo et al., 1988). Our results indicate that the molar ratio of thujaplicin to copper ions was about 2:1 at the point where the virus-induced apoptosis was completely inhibited, suggesting that the anti-apoptosis effect is due to the function of the copper chelate itself on the virus-infected MDCK cells, but not to chelation of intercellular metal ions by free thujaplicins in culture medium.

Next, we examined the inhibitory effects of the copper chelates on the release of viruses from the infected MDCK cells during apoptosis. Both the HA titers and the titers of virus significantly

decreased not only in the supernatants after treatment of the infected MDCK cells with each thujaplicin–copper chelate, but also in the supernatant after infection of MDCK cells with the influenza virus for 3 h in the presence of each thujaplicin–copper chelate (Table 3). These results suggest that thujaplicin–copper chelates affect MDCK cells directly in the early stage of influenza virus-induced apoptosis and also inhibit apoptosis and the release of influenza viruses from the infected MDCK cells. In addition, we examined the effect of time of addition of the copper chelate on virus-induced apoptosis and the results indicate that the copper chelates inhibited the induction of cell death during the early stage of infection, occurring 2–4 h postinfection (Fig. 3). Takizawa et al. (1993, 1995) demonstrated that there might be a critical period for the induction of cell death during the early stage of infection, occurring 2–4 h postinfection. Our results in this study also indicate that the anti-apoptotic effects of the copper chelates may be associated with the early stage of infection, suggesting that the copper chelates might inhibit induction or activation of a host protein(s) that triggers the apoptosis or, alternatively, small amount of viral proteins that had been produced before the addition of the copper chelates at 4 h postinfection, during the early phase of viral growth. Thujaplicin–copper chelates would be potent anti-influenza viral agents to inhibit apoptosis induced in the virus-infected host cells and also to prevent expansion of influenza viral infection.

Recent reports suggested that the cytotoxic effect of β-thujaplicin may be due to the inhibition of DNA synthesis (Okabe et al., 1988, Inomori et al., 1993, Muto et al., 1995). Yamato et al. (1986) suggested that tropolone and its derivatives may inhibit ribonucleotide reductase, an obligatory enzyme in DNA synthesis. They proposed that β-thujaplicin chelates ferric ions present in the catalytic moiety of this enzyme leading to its inactivation. In addition, Kontoghiorghes et al. (1986) reported the cytotoxic and DNA-inhibitory effects of some iron chelators. As we used the metal chelates themselves in this study, the inhibitory effects of thujaplicin–copper chelates on the virus-induced apoptosis might not be due to chelation of metal ions in host cells by thujaplicins.

Hinshaw et al. (1994) suggested that apoptosis may be a general mechanism of cell death in hosts infected with influenza viruses, and demonstrated that expression of *bcl-2* reduced the levels of infectious virus production, and this effect was associated with modified glycosylation of the viral hemagglutinin protein (Olsen et al., 1996). On the other hand, Takizawa et al. (1993, 1995) suggested that activation of the Fas antigen gene in the early phase of infection is an important event for apoptosis induced in virus-infected HeLa cells, and that it is regulated by the double-stranded RNA–interferon system involving protein phosphorylation. Thujaplicin–copper chelates exhibited the inhibitory effects on the virus-induced apoptosis also in HeLa cells, and the expression of Fas antigen in the virus-infected HeLa cells was inhibited significantly by the addition of the copper chelates whithin 4 h by FACS analysis (unpablished data). These data suggest that the copper chelates may inhibit induction of Fas antigens or activation of a protein(s) related to Fas-mediated apoptosis, such as interleukin-converting enzyme-like and CPP32-like proteases, in the virus-infected cells. Further analysis of the mechanisms by which thujaplicin–copper chelates block virus-induced apoptosis would contribute to the development of anti-influenza virus drugs that could prevent expansion of viruses in tissue, and the elucidation of the mechanisms underlying virus-induced apoptosis.

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