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Antiviral Research 37 (1998) 37–45

Antiviral Research

Inhibitory effect of furanonaphthoquinone derivatives on the replication of Japanese encephalitis virus

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Received 14 April 1997; accepted 3 October 1997

Abstract

Japanese encephalitis still occurs in endemic and epidemic forms over a wide area of Asia. Although the vaccine against Japanese encephalitis virus (JEV) is widely used, no antiviral drug has been reported. We used several different kinds of furanonaphthoquinone derivatives and found antiviral activity against JEV. Especially, 2-methyl-naphtho[2,3-*b*]furan-4,9-dione (FNQ3) indicated the highest antiviral activity, followed by 2-(1-hydroxyethyl)-, 5(or 8)-hydroxy-, and 2-methyl-5(or 8)-hydroxy-analogs of naphtho[2,3-*b*]furan-4,9-dione. In the presence of 3 μg/ml FNQ3, the virus yields in Vero cells were 2×10^5 PFU/ml at 24 h after infecting with the virus and 10% of the control level. Western blot analysis using anti-E rabbit sera or anti-NS3 showed that the expression of viral proteins was inhibited by treatment with FNQ3. In addition, Northern blot analysis indicated that the appearance of JEV-RNA was also inhibited by FNQ3. These results suggest that FNQ3 inhibits JEV replication through viral RNA and protein synthesis. © 1998 Elsevier Science B.V.

Keywords: Japanese encephalitis virus; Furanonaphthoquinone; Replication

1. Introduction

Japanese encephalitis virus (JEV) is a member of the Flaviviridae family and has a positive, single strand RNA genome about 11 kb in length (Sumiyoshi et al., 1987; Hashimoto et al., 1988). JEV is a human pathogenic flavivirus and is widespread in south-east Asia, China, Korea and Japan. Japanese encephalitis caused by JEV still occurs in endemic and epidemic forms in a wide area of Asia (Monath et al., 1986; Igarashi, 1993). Although the vaccine is used to protect against JEV infection, the relatively high cost of the immunization is a problem. Accordingly, the development of cheap antiviral drugs against JEV is

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PII S0166-3542(97)00058-2

expected. So far, no antiviral drugs except interferon (INF) have been reported. Therefore, it is very important to develop a new antiviral drug against JEV.

The furanonaphthoquinone-derivative, 5-hydroxy-2-(1-hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione (FNQ7), has been purified from the inner bark of *Tecoma ipe* Mart, *Tabebuia impetiginosa*, *T. barbata*, *T. avellanedae*, and so on, and is known to have possible antitumor (Inoue et al., 1981; Rao and Kingston, 1982; Fujimoto et al., 1991; Ikekawa et al., 1985) and antimicrobial activity (Binutu and Lajubutu, 1994). We synthesized several isomeric derivatives and found that seven of them had an antitumor action spectrum that was as good as that of FNQ7 in bioassays using human malignant tumor cells (Hirai et al., 1995) and in vivo assays using nude mice (Pan et al., 1997). It was shown that these reagents had selective activities, since their lethal doses for cancer cells were 3–8.5 times higher than those for normal cells. Pan et al. reported that FNQ3 was selectively toxic to a cancer cell line. Its toxicity was approximately 8.5 times stronger to HeLa cells than to normal cervical cells, also inducing necrosis in HeLa cells by mechanisms involving mitochondrial alteration and free radical formation (Pan et al., 1997). It was expected that FNQ might be an antiviral reagent without side effects. In this report, we checked the antiviral activity of FNQ and found that FNQ had an inhibitory effect on JEV replication.

2. Materials and methods

2.1. Preparation of FNQ

FNQ3 and 2-(1-hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione (FNQ4) were prepared by the method of Lee (Hayashi et al., 1987) and Koyama (Koyama et al., 1987), respectively. A mixture of 1 g 3-hydroxyphthalic anhydride, 0.7 g 2-acetylfuran and 2.5 g aluminum chloride in 5 ml nitrobenzene was heated at 100°C for 18 h, and following the above procedure, 5(or 8)-hydroxynaphtho[2,3-*b*]furan-4,9-dione (FNQ12) was obtained. By this procedure, 2-methyl-5(or 8)-hydroxynaphtho[2,3-*b*]furan-4,9-dione (FNQ13) was synthesized using 2-acetyl-5-methylfuran. The purity of crystallized FNQ was close to 100% which was estimated by NMR. FNQ is stable in the store at the room temperature under the shading, while FNQ dissolved in the solution dimethyl sulfoxide is gradually degraded.

2.2. Viruses and cells

The JEV JaGAr-01 strain was used in the study. The monkey kidney cell line Vero was adsorbed with JEV for 1 h at ten multiplicity of infection (m.o.i.). After the removal of virus solution, cells were cultured in MEM containing 5% FCS for 24 h. Viruses in cultured fluids were titrated by the plaque method using BHK cells as previously described (Takegami and Hotta, 1990). The infected cells were harvested, centrifuged and cell pellets were suspended in TE buffer (Tris–HCl 50 mM, pH 8.0, EDTA 1 mM). Cell extracts were used for the preparation of proteins and RNAs.

2.3. Inhibition test of virus reproduction by FNQ

After the adsorption with JEV, Vero cells were added with various concentrations of FNQ and cultured for 24 h. Virus titers in the culture fluids were estimated by the plaque method. On the other hand, the direct effect of FNQ on virus particles was examined as follows. Viruses were mixed with FNQ and incubated for 1 h at 37°C. Then, those viruses were used for the infection to Vero cells, and virus yields at 24 h post infection were examined.

2.4. Cytopathic effect on cells

Cytopathic effect by reagents and viruses on cells was estimated by the morphological change and the influence to cell growth. Cell growth was examined by the culture system using 96-well plate and staining with crystalviolet, as previously described (Pan et al., 1997).

2.5. Antisera and JEV-riboprobe

The monospecific rabbit antisera, anti-NS3 and anti-E, were prepared as previously described (Takegami et al., 1982; Zulkarnain and Takegami, 1993). The JEV-riboprobe was synthesized by the method of Takegami et al. (1994). Here, digoxigenine labeled UTP was used for the preparation of the JEV-riboprobe.

2.6. Western blot analysis

The protein sample (20 μg) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and transferred to a membrane, Immobilon P (Millipore, USA). It was reacted with the anti-NS3 or anti-E in the first reaction and then incubated in an anti-rabbit IgG goat serum conjugated with peroxidase. Finally, the reacted protein was visualized by incubation with diaminobenzidine. Each specific band was measured by a Flying spot scanning densitometer CS-9000 (Shimadzu, Japan).

2.7. Northern blot analysis

RNA was extracted by the phenol method using Isogen (Nippongene, Japan). RNA extracts were analyzed by electrophoresis using 1% denaturing agarose gel (0.1 M MOPS, 30 mM sodium acetate, 5 mM EDTA, 0.5% formaldehyde) and transferred to a nylon membrane (Millipore, USA). The membrane was hybridized with JEV-riboprobe labeled with digoxigenine. It was then reacted with anti-digoxigenine conjugated alkaline phosphatase, after which JEV-RNAs were detected by NBT.

3. Results

3.1. The effect of FNQ derivatives on virus growth

All of the FNQ derivatives had an inhibitory effect on JEV growth (Table 1). The antiviral activities of FNQ3 and FNQ13 were relatively higher than those of FNQ4 and 12 (Table 1). Although FNQ13 treatment showed cytopathic effect on the Vero cells, FNQ3 did not indicate any toxicity under the same conditions. These results indicate that the cytopathic effect of FNQ was due to the chemical structure and concentration of reagent (Fig. 1).

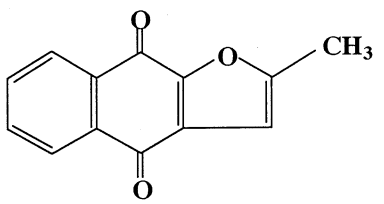
3.2. The inhibitory effect of FNQ3 on JEV growth

To analyze the mechanism of FNQ antiviral activity, we selected FNQ3 and examined the inhibitory effect on virus growth at the different doses (1–10 μg/ml). The inhibition of the virus growth was dose-dependent and virus yields at 24 h in the presence of 10 μg/ml FNQ3 decreased to 2.0% of the control level (Table 2). The similar experiments using FNQ3 were repeatedly performed. The virus yields at 24 h decreased to 10–20% of the control by treating with FNQ3 (1–9 μg/ml) and to 2% by 10 μg/ml FNQ3 (Fig. 2). Under these conditions, the FNQ3 treatment did not indicate any cytopathic effect on the Vero cells. JEV-infected Vero cells showed a remarkable cytopathic effect (Fig. 3b), but the FNQ3 treatment changed their morphology and showed features similar to those seen in the uninfected cells (Fig. 3a and c). The growth rate of Vero cells in the presence of FNQ3 at 1–10 μg/ml was not different from the control level (data not shown). However, FNQ3 at a much higher concentration (20 μg/ml) was very toxic to the Vero cells (data not shown). Accordingly, FNQ3 at lower concentration seems to influence the virus particles or virus formation in the cells, directly or indirectly.

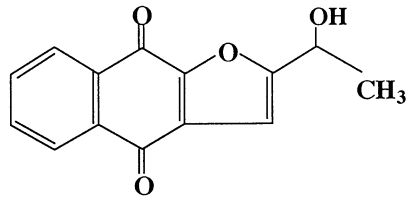
Table 1
The effect of furano-napthoquinone dervative on JEV

Reagents	Conc. (μg/ml)	PFU/ml[a]	Growth (%)	CPE[b]
None	0	2.1×10^6	100.0	+
FNQ3	3.3	2.0×10^5	9.8	−
FNQ4	3.3	5.2×10^5	24.6	−
FNQ12	3.2	2.9×10^5	13.7	+
FNQ13	3.6	1.3×10^5	6.1	++

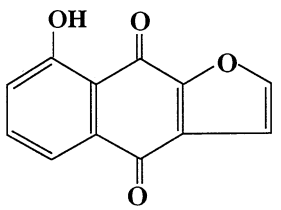
[a] The virus amounts after 24 h.
[b] Cytopathic effect.



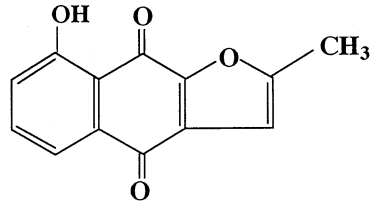
2-Methylnaphtho[2,3-*b*]furan-4,9-dione (FNQ3)



2-(1-Hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione (FNQ4)



5(or 8)-Hydroxynaphtho[2,3-*b*]furan-4,9-dione (FNQ12)



2-Methyl-5(or 8)-hydroxynaphtho[2,3-*b*]furan-4,9-dione (FNQ13)

Fig. 1. Structure of furanonaphthoquinone derivatives.

To examine the effect of FNQ3 on virus particles, JEV was preincubated with FNQ3 at various concentrations at 37°C for 1 h. However, the infection and growth of JEV pretreated with FNQ3 were not different from those of the control JEV (Fig. 4).

3.3. The analysis of virus proteins by Western blot

Next, the influence of FNQ3 on the expression of viral proteins E and NS3 was examined. The expression of E protein in JEV-infected cells decreased by FNQ3 treatment dose-dependently (Fig. 5). Another protein, with a molecular weight of about 48 kDa which existed in JEV-infected cells and reacted with anti-E, disappeared with the FNQ3 treatment. Interestingly, a protein with a molecular weight of 50 kDa was detected in the cells treated with a relatively higher dose of FNQ3 (7–10 μg/ml). The amount of JEV-nonstructural protein NS3 in the infected cells also decreased by FNQ3 treatment (Fig. 6a), but the decrease did not depend on FNQ3 concentration. It differed from that of the E protein. The amounts of each protein were estimated by densitometer and then confirmed (Fig. 6b). The contents of E and NS3 proteins decreased to 55.4 and 26.7% of the control level by the FNQ3 treatment of 1 μg/ml. However, the expression of NS3 protein was 13.1% by the FNQ treatment of 10 μg/ml while the level of E protein was 2% of the control level under the same conditions.

Table 2
The effect of FNQ3 on JEV growth

Conc. (μg/ml)	PFU/ml[a]	Growth (%)	CPE[b]
0	7.9×10^5	100	+
0.1	2.8×10^5	35.4	−
0.5	2.4×10^5	30.4	−
1.0	1.3×10^5	16.4	−
3.3	7.7×10^4	9.8	−
10.0	1.6×10^4	2.0	−
20.0	1.2×10^4	1.5	+

[a] The virus amounts after 24 h.
[b] Cytopathic effect.

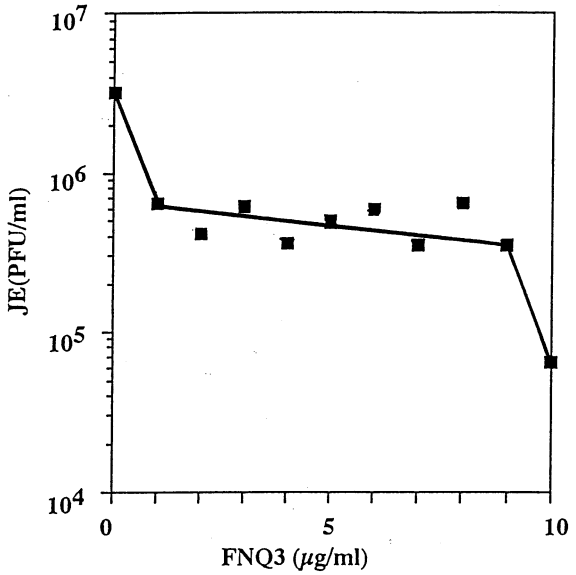


Fig. 2. The effect of FNQ3 on JEV replication. Vero cells were infected with JEV at 10 m.o.i. and cultured in the presence of FNQ3 at 1–10 μg/ml. Virus titer in the culture fluids was assayed by the plaque method using BHK cells.

3.4. Decrease of JEV-RNA by FNQ3 treatment

In order to examine the effect of FNQ on the appearance of JEV-RNA in the infected cells, a specific RNA probe which can detect only positive strands of JEV-RNA (42S) was used. Sample RNA was extracted from the JEV-infected cells that had been cultured for 24 h in the presence or absence of FNQ3 at 5 μg/ml. As shown in Fig. 7, lane 3, the appearance of JEV-RNA (indicated by arrowhead) was inhibited by the treatment of FNQ3. The amounts of total RNA, mainly ribosomal RNA from FNQ-treated cells were not so different from other samples. That is, the amounts of JEV-RNA in FNQ-cells (lane 3) were very low despite of so much total RNA content.

4. Discussion

Much attention has been paid recently to the anticancer properties of FNQ but so far the antiviral activity of FNQ has not been reported. To check the antiviral activity of FNQ, a JEV repli cation system was used. As shown in the present

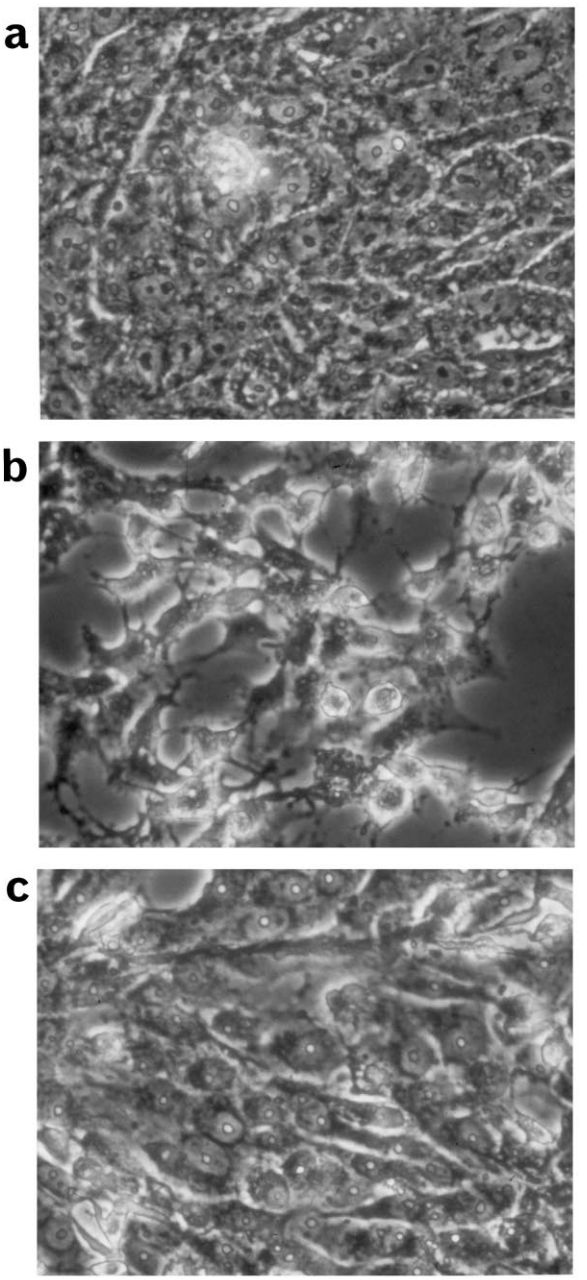


Fig. 3. Cytopathic effect on Vero cells by the treatment of FNQ3. (a) Vero cells as control. (b) JEV-infected Vero cells cultured for 24 h in the presence or (c) absence of FNQ3, 3.3 μg/ml.

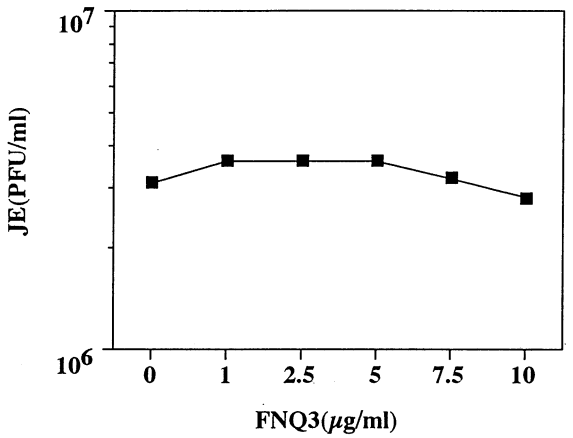


Fig. 4. Effect of FNQ3 on the activity of JEV virion. JEV virions were incubated with MEM containing FNQ3 1–10 μg/ml (serum free) at 37°C and then adsorbed into the BHK cells. The virus titer in the culture fluids was assayed as described in the legend to Fig. 2.

results, FNQ inhibited JEV replication. As to the mechanism of the inhibitory effect of FNQ on JEV replication, there are several possibilities. At first, we checked whether FNQ directly attacked the JEV virion or not. The results indicated that JEV growth was not influenced by pretreatment with FNQ at various concentrations. Taken together with the results when FNQ was present in the culture medium, the JEV yield was obviously inhibited, FNQ seemed to function on the host cell, making an antiviral state. On the other hand, FNQ did not show any cytopathic effect on the host Vero cells when the drug was under a physiological concentration. This confirmed the previous report that FNQ showed severe cytopathic effects on the tumor cells but not on normal cells (Pan et al., 1997). Although the mechanism of anti-tumor by FNQ is not so clear, it is one of the possibilities that the higher activity of cellular metabolism including mitochondria functions in the tumor cells is affected by FNQ treatment (Pan et al., 1997). In the virus infected cells, an abnormal stage appears and seems to be fit for viral replication. FNQ could work to eliminate such an abnormal situation of which some parts are similar to the cancer cells. This effect of FNQ seems to be dependent on chemical structure and concentration of reagents. As shown in the case of

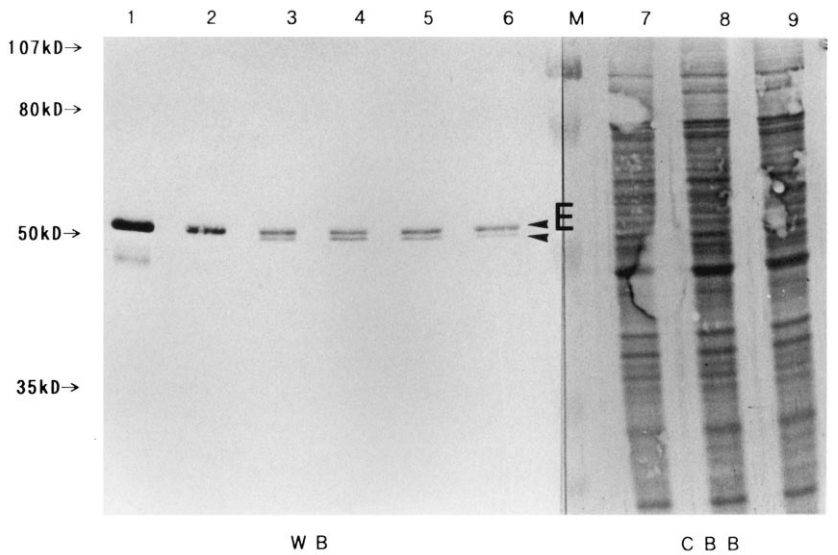


Fig. 5. Detection of JEV specific proteins by Western blot. Vero cells were infected with JEV and cultured in the presence of FNQ3 for 24 h. Cell extracts were subjected to SDS–PAGE and then transferred to the membrane, Immobilon P. They were reacted with specific antisera against E protein, anti-E (left panel): lane 1 is the control and lanes 2 through 6 indicate FNQ3 treatment at 6, 7, 8, 9 and 10 μg/ml, respectively. Part of the membrane was stained with 0.05% coomassier brilliant blue (right panel): lanes 7, 8 and 9 indicate FNQ3 at 0, 7 and 9 μg/ml, respectively. Arrowheads show the JEV-specific E and E-related proteins. Molecular size markers for proteins (Bio Rad) were used and indicated on the left.

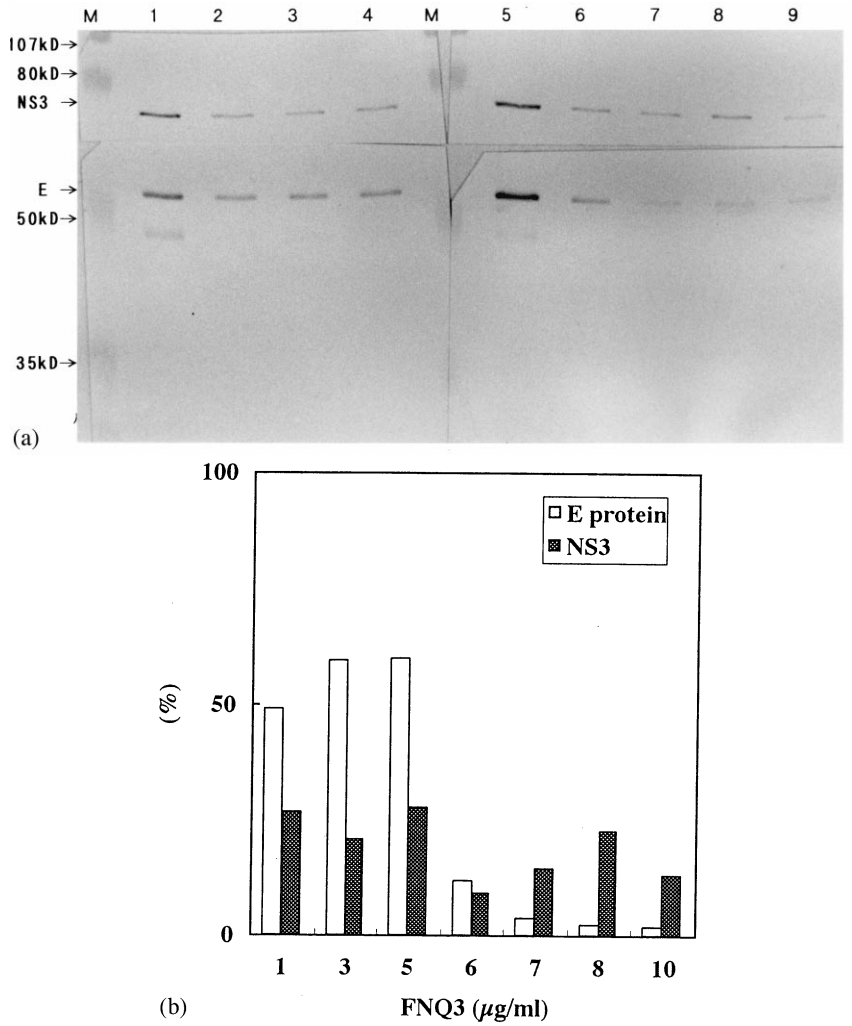


Fig. 6. Detection of JEV specific proteins, NS3 and E, by Western blot. After infection with JEV, Vero cells were cultured in the presence of FNQ3 at 1–10 μg/ml for 24 h. (a) Cell extracts were subjected to SDS–PAGE and Western blot analysis using anti-NS3 and anti-E. Lanes 1 and 5 are the controls. Lanes 2, 3, 4, 6, 7, 8 and 9 indicate the treatment by FNQ3 at 1, 3, 5, 6, 8, 9 and 10 μg/ml, respectively. (b) All bands were measured by a densitometer and are shown as percentages of the control level.

FNQ3, concentration for antiviral effect without morphological change in the cells is limited and not so different between 1–9 μg/ml. This might be related with the mechanism of antiviral effect of FNQ on the cells.

The possibility that FNQ affects the process of JEV replication in infected cells was examined. We obtained data that FNQ inhibited the expression of viral proteins and also genomic RNA. In addition, the expression of envelope protein E was much more inhibited than that of viral nonstructural protein NS3. In the influenza virus-infected cells, we also detected that the expression of viral surface protein HA was clearly inhibited and the decrease was much higher than that of other viral proteins (Hatada et al., unpublished data). Interestingly, the replication of poliovirus, an unenveloped virus, was not influenced by FNQ treatment (Ago et al., unpublished data). These results suggest that the process of viral envelope protein

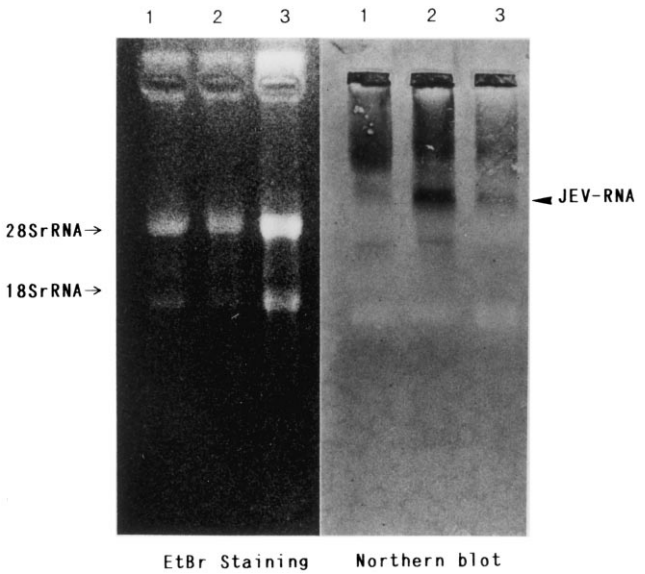


Fig. 7. Detection of JEV-RNA in the infected cells Vero cells were infected with JEV and cultured in the presence of FNQ3 at 5 μg/ml. RNAs were extracted and subjected to 1.2% agarose gel. Left and right panels show the staining with EtBr and Northern blot, respectively. After the electrophoresis, RNA samples were transferred to nylon membrane and hybridized with JEV-riboprove labeled with digoxigenin. Lane 1 shows the control Vero cells. Lanes 2 and 3 indicate RNAs from JEV-infected Vero cells cultured in the absence and presence of FNQ3.

formation might be inhibited by FNQ in the host cells. The study on this working hypothesis is under the progress in our laboratory.

Although antiviral drugs such as IFN (Sakai et al., 1995), Ribavirin (Brown and Condreay, 1986) and Amantadine (Dolin, 1988) have been reported, they have severe side-effects. From the fact that FNQ3 did not show any cytopathic effects on Vero cells, it is very possible that FNQ could be used as an antiviral drug in clinical treatment. Recently we also found that suramin, an antitrypanosoma drug, had good antiviral activity against JEV replication (Xu and Takegami, 1996). Such a trial seems to be important in the development of new antiviral drugs. In the near future, it will be very possible to use a new drug against flavivirus infections including Hepatitic C virus (HCV), which causes chronic hepatitis and hepatocellular carcinoma in a different mechanism (Sakamuro et al., 1995). For this aim, it is essential to investigate the mechanism of FNQ antiviral activity.

Acknowledgements

We are grateful to Ke-Shu Xu and Yoko Funamoto for their technical assistance. This work was partly supported by grants from Kanazawa Medical University Project Research (P95-18), Tokyo Metropolitan Institute and Ministry of Education, Japan(08670352).

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