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Short Communication

Inhibitory effect of bafilomycin A1, a specific inhibitor of vacuolar-type proton pump, on the growth of influenza A and B viruses in MDCK cells

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Abstract

We studied the effect of bafilomycin A1 (Baf-A1), a novel and highly specific inhibitor for vacuolar-type proton ($V\text{-}H^+$) pump, on the growth of influenza A and B viruses in Madin–Darby canine kidney cells. Vital fluorescence microscopic study showed that Baf-A1 induced the complete disappearance of acidified compartments such as endosomes and lysosomes both in infected and uninfected cells by the treatment with 0.1 μM inhibitor for 1 h at 37°C. In addition, virus growth was inhibited when Baf-A1 was present from 1 h before infection to the end of incubation, or added within as early as 5–10 min after infection. Conversely, the virus growth was recovered in correlation with the reappearance of acidified compartments after removal of Baf-A1. These data suggest that Baf-A1-sensitive $V\text{-}H^+$ pumps are solely responsible for the acidification of endosomes and lysosomes, and thus Baf-A1 inhibits the growth of influenza A and B viruses by affecting the acidified compartments in which low pH is essential for the uncoating process of influenza virus growth at an early stage of infection.

Keywords: Influenza virus; Bafilomycin A1;Vacuolar type-proton pump;Acid-catalyzed fusion reaction

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It has been shown that the acidic condition in endosomes and lysosomes (referred to as ELS in this study) is essential for the uncoating process of influenza virus infection by triggering the viral envelope fusion activity (for review, see White et al., 1983). Indeed, chloroquine, an endosomotropic weak base, inhibits influenza virus growth by raising the pH within ELS (Shibata et al., 1983). On the other hand, several studies indicate that the ELS membranes contain vacuolar-type proton ($V\text{-}H^+$) pumps, which are, at least in part, responsible for the acidification of ELS (Ohkuma et al., 1982, Forgac et al., 1983). Considering the ion channel activity of influenza virus (Pinto et al., 1992), the question arises as to whether acidification of ELS is regulated by the contribution of additional proton transfer systems induced in response to influenza virus infection. The use of bafilomycin A1 (Baf-A1), a novel and highly specific inhibitor of the $V\text{-}H^+$ pumps (Werner et al., 1984, Bowman et al., 1988, Hanada et al., 1990), allows us to examine this issue by overcoming the disadvantage of chloroquine. Thus, we examined the relation of drug effect between acidification of ELS and virus growth in Madin–Darby canine kidney (MDCK) cells. The culture condition of the cells, preparation of virus solution and assay method of hemagglutinin (HA) titers were described previously (Ochiai et al., 1988).

After treatment with various doses of drug at 37°C for 1 h (short-term treatment), the cells were processed for vital fluorescence staining (Yoshimori et al., 1991) with acridine orange, an acidotropic weak base, which is taken up by living cells and changes its color from green at low concentrations to orange at the accumulated sites such as ELS (Allison and Young, 1969, Holtzman, 1989). With the aid of a fluorescence light microscopy (Fig. 1), numerous acidified compartments with a dotted distribution could be observed in the drug-untreated cells. Their number was decreased in the drug-treated cells in a dose-dependent manner. At 0.1 and 0.05 μM, Baf-A1 inhibited completely and partially the acidification of ELS, respectively, whereas the effect of 0.025 μM Baf-A1 was negligible. In the cells treated with 0.1 μM Baf-A1, only diffused green fluorescence was observed clearly in the nuclei and faintly in cytoplasm. In the long-term treatment (for 3 days), drug effect was slightly enhanced, that is, the effects of 0.05 and 0.025 μM Baf-A1 became comparable to those of 0.1 and 0.05 μM, respectively, in the short-term treatment, but the effect of 0.125 μM was limited (data not shown). On the other hand, in the cells infected with A/NWS (H1N1), A/Aichi (H3N2) or B/Lee virus, the appearance of acidified compartments was also inhibited at a level comparable to that in the uninfected cells, suggesting that Baf-A1-sensitive $V\text{-}H^+$ pumps are solely responsible for the acidification of ELS, even in the infected cells. The representative figures for NWS virus-infected cells are shown in Fig. 1.

As shown in Table 1, the growth of 3 virus strains mentioned above was inhibited in the presence of drug from 1 h before infection to either 8 h (for A viruses) or 10 h (for B virus) postinfection (p.i.) in a dose-dependent manner. In the control culture supernatants collected after 3 times of freezing and thawing, titers of 64–256 HA were detected, whereas these values decreased proportionally in those of cells treated with 0.1 and 0.05 μM Baf-A1. At 0.025 μM, Baf-A1 showed no growth-inhibitory effect on either virus. Conversely, when the cells infected with NWS virus were initially treated with 0.1 μM Baf-A1 from 0 to 1 h p.i., and then cultured further in drug-free media, acidification of ELS recovered with a time-lag of 2 h (data not shown). In correlation with the

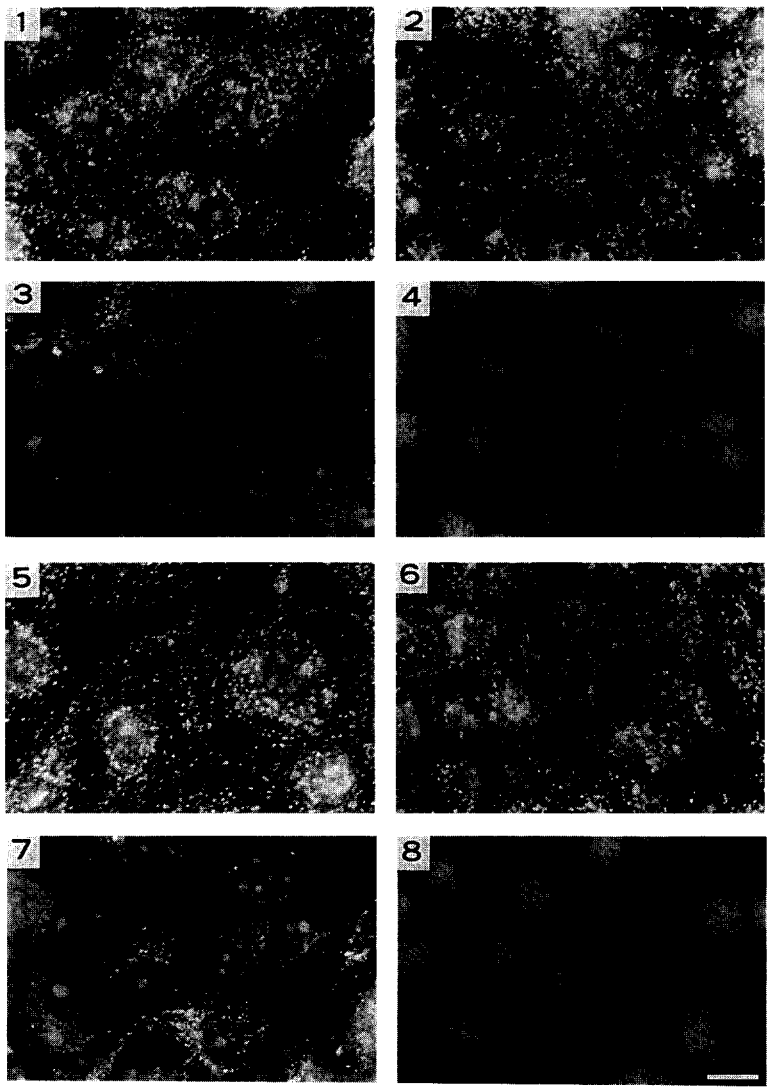

Fig. 1. Effect of Baf-A1 on the acidification of ELS in MDCK cells infected with or without influenza A NWS virus. Confluent monolayers of the uninfected cells (1–4) and infected cells (5–8) on the cover slips in a 24-well plate were washed once with phosphate-buffered saline and then treated with 0.025 μM (2 and 6), 0.05 μM (3 and 7) or 0.1 μM (4 and 8) Baf-A1, or not (1 and 5) in serum-free media for 1 h at 37°C. For the virus infection, the cells, either treated previously or not with a drug, were infected with NWS virus at a multiplicity of infection of 5 plaque forming units/cell for 45 min at room temperature followed by washing 3 times as above, and then cultured for 1 h at 37°C in the presence of the same drug doses as for the previous treatment. Thereafter, the cells were processed for vital fluorescence microscopy. The representative figures of 3 experiments are presented. Bar represents 20 μm.

Table 1
The effect of Baf-A1 on the growth of influenza A and B viruses in MDCK cells

Virus	Incubation period (h)	HA titers in culture supernatants in the presence of the indicated drug concentrations (μM) [a]			
		0.1	0.05	0.025	(−)
A/NWS	8	< 4	16	64	128
A/Aichi	8	4	32	128	256
B/Lee	10	< 4	8	32	64

[a] Baf-A1 was present from 1 h before infection to the end of cultures. The representative data from 3 experiments are shown.

reappearance of acidified compartments, the virus growth was also recovered with a time-lag of 3 h (Fig. 2). These findings show that the drug effect was reversible, and unfused virus particles under drug treatment were protected from hydrolytic degradation in lysosomes containing acidophilic enzymes in accordance with the previous report (Yoshimori et al., 1991). Table 2 shows that the virus growth was inhibited by the initiation of drug treatment (0.1 μM) within as early as 5–10 min p.i. depending on the virus strains. The time-related drug effect was almost equal to that of chloroquine, confirming that both drugs affect the early phase of influenza virus infection. Under multiple-cycle growth conditions in the agar media (Fig. 3), the efficiency of plaque formation was almost equal between control and 0.125 μM-containing cultures from the aspects of plaque number and size. The presence of 0.25 μM Baf-A1 resulted in the production of tiny plaques (1.0–1.5 versus 3–4 mm in diameter) with slightly less plaques, and no plaque formation was observed at 0.5 μM. Considering the data of the

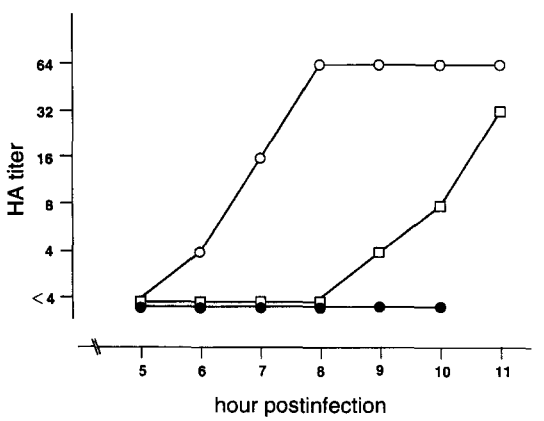

Fig. 2. Reversible effect of Baf-A1 on the virus growth of NWS virus in MDCK cells. Confluent monolayers in a 24-well plate were infected with NWS virus and then cultured in the presence of drug for 1 h at 37°C as described in the legend to Fig. 1. Thereafter, the cells were further cultured in 0.5 ml of drug-containing (solid circle) or drug-free media (open square) at 37°C. The control cells were processed throughout in the drug-free condition (open circle). At the indicated time post infection (abscissa), the culture supernatants were collected to assay HA titers (ordinate). The representative figures of 3 experiments are presented.

Table 2
Time-related drug effect on the growth of influenza A and B viruses in MDCK cells

Virus	Drug [a]	HA titers in culture supernatants [b] by the indicated initiation of drug treatment					
		0	5	10	15	20 min p.i.	Control [c]
A/NWS	Baf-A1	< 4	4	64	64	128	128
	Chloroquine	< 4	< 4	< 4	32	64	
A/Aichi	Baf-A1	< 4	< 4	16	32	64	256
	Chloroquine	< 4	< 4	< 4	16	128	
B/Lee	Baf-A1	< 4	< 4	4	8	32	64
	Chloroquine	< 4	< 4	16	16	32	

[a] Concentrations of Baf-A1 and chloroquine were 0.1 μM and 100 μg/ml, respectively.
[b] Culture supernatants were collected at 8 and 10 h p.i. for A and B viruses, respectively.
[c] Control indicates the cultures processed throughout in drug-free medium. The representative data from 3 experiments are presented.

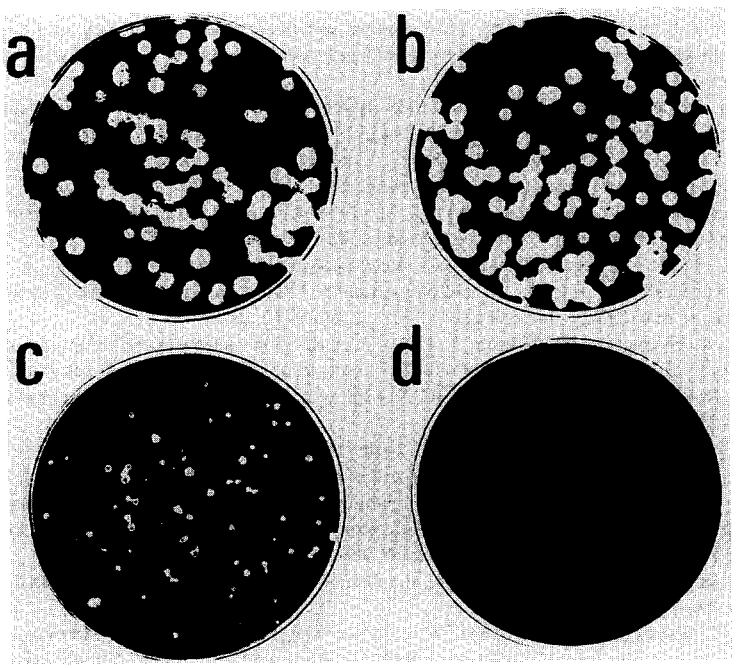

Fig. 3. Growth inhibitory effect of Baf-A1 in MDCK cells under multiple-cycle growth conditions. Confluent monolayers in a 6-cm dish were inoculated with 0.2 ml of NWS virus solution with the infectivity of about 100 plaque forming units and then cultured in the agar media without drug (a) or with 0.0125 μM (b), 0.025 μM (c) or 0.05 μM (d). Three days later, the cells were fixed with 5% formalin and stained with 0.01% Crystal violet to visualize plaques. The representative figures of 3 experiments using triplicate cultures for each experimental point are presented.

long-term treatment (see above), the drug effects on virus growth and acidified ELS might be similar under single and multiple-cycle growth conditions.

As to cytotoxicity, 1 μM Baf-A1 seemed to induce cell atrophy and nuclear pycnosis even in the short-term treatment, but toxicity following 10 h treatment with 0.1 μM could be ruled out, judging from Trypan blue exclusion test and re-growth assay of drug-treated cells in drug-free media (data not shown). In the long-term treatment, the cytotoxic activity of 0.05 μM Baf-A1 could be also ruled out, judging from the density of the remaining cells stained with Crystal violet (see Fig. 3).

In summary, it has been demonstrated that Baf-A1 inhibits the growth of influenza A and B virus showing a strict relation with the acidification of ELS. These findings also suggest that Baf-A1-sensitive V-H^+ pumps are solely responsible for the acidification of ELS, eliminating the possibility of the induction of Baf-A1-insensitive proton transfer systems in the influenza virus-infected cells. Baf-A1 may be a useful tool to study the uncoating process in certain virus infections. We are also investigating other possible mechanisms of anti-influenza drugs, such as ion channel inhibitors.

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