Non-Sialate Inhibitor of Influenza A/WSN/33 Neuraminidase

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ABSTRACT: An N_1 strain of influenza A virus neuraminidase (A/WSN/33 NA) was purified and used to screen for inhibitors. As a result, a well-known tuberculostatic, 4′-formylacetanilide thiosemicarbazone (or thiacetazone), was identified. Thiacetazone is a non-sialate compound and inhibits the enzyme in a noncompetitive manner with respect to the substrate sialic acid. Mechanistic studies indicate that the inhibition was due to the competition of thiacetazone with Ca^{2+}, which maintains N_1 neuraminidase in an active conformation. The K_i for the inhibition was estimated to be about 4 μM. Equilibrium exchange experiments revealed that when purified A/WSN/33 NA was incubated with 5 μM $^{45}CaCl_2$, 2 mol of $^{45}Ca^{2+}$ ion was exchanged into each mole of NA tetramer and subsequently displaced from the enzyme upon the introduction of the inhibitor. Inhibition of plaque formation by thiacetazone in an MDCK cell culture that had been infected with the influenza A/WSN/33 virus was demonstrated. Thiacetazone was highly specific for A/WSN/33 neuraminidase, since little effect was noted when it was tested against NAs from the other strains of influenza virus or from bacteria. This compound might represent a group of non-sialate inhibitors of influenza NA that bind to a noncatalytic or an allosteric site on the enzyme.

Influenza virus is one of the most important but poorly controlled human pathogens. Some flu outbreaks in this century have caused serious pandemics and resulted in significant loss of life [for review, see Webster et al. (1993), Smith and Palese (1989), and Potter (1992)]. From a molecular and cellular biology standpoint influenza is one of the best-studied viruses [for review, see Katze and Krug (1990) and Lamb (1989)], yet little progress has been made in combating the disease. Although flu vaccines have been used selectively, particularly for the elderly and high-risk groups, the hypermutability of the virus has been a major obstacle that limits the extensive application of vaccines to the general public. Currently, new vaccines need to be formulated each year on the basis of WHO's best guess of what antigenic determinants are likely to emerge in the next outbreak. Since scale-up of the vaccines for clinical use requires at least 8 months of time, strain prediction thus must be made far in advance of any epidemic. Inevitably, the vaccine has sometimes been mismatched with the epidemic strain (Beyer et al., 1993).

Alternatively, amantadine and its structural analogue rimantadine have also been demonstrated to be effective in inhibiting virus replication [Dolin et al., 1982; Wingfield et al., 1969; Kato & Eggers, 1969; for review, see Monto and Arden (1992), Hay (1992), and Helenius (1992)]. It was recently reported that the alteration of the M_2 channel conductivity by these compounds was the underlying mechanism of inhibition (Pinto et al., 1992). Unfortunately, the rapid emergence of resistant strains (Hayden et al., 1989) and both GI and CNS adverse effects, perhaps due to unanticipated blocking of endogenous ion channels such as the *N*-methyl-D-aspartic acid receptor-gated ion channel (Kornhuber et al., 1991; Stoof et al., 1992), limit their effectiveness and therapeutic application.

The influenza neuraminidase (NA) is one of the best-characterized viral enzymes at the molecular level (Air et al., 1989; Varghese et al., 1983; Bossart-Whitaker et al., 1993). Its enzymatic function is to catalyze the hydrolysis of terminal *N*-acetylsialic acid from glycoconjugates on the cell surface. It is generally agreed that viral NA plays a pivotal role in allowing the progeny virus to be released from the surface of infected cells, as well as in preventing self-aggregation of the virus (Griffin & Compans, 1979, 1983; Liu & Air, 1993; Schulman & Palese, 1977). The enzyme may also assist in virus spreading and access to new cells by cleaving sialic acid from the mucins that overlay the epithelial cells of the respiratory tract (Klenk & Rott, 1988; Burnet & Stone, 1974). It is, therefore, plausible that the inhibition of viral NA might retard the mobility of the virus and confine the progeny virions to the infected cell surface. Consequently, the spread of the freshly budded virions might be controlled, and the restricted progeny would be an ideal antigen for the immune response.

The early work of Meindl, Palese, and co-workers involving the synthesis of sialic acid analogues led them to identify the first NA transition state inhibitor, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Meindl et al., 1974; Palese et al., 1974). Recent crystallographic data obtained for influenza NA enabled von Itzstein et al. to design a new generation of transition state inhibitors for this enzyme (von Itzstein et al., 1993). Although such inhibitors, which target the active site of the enzyme, should minimize the emergence of resistant strains, they may also be structurally recognized as substrates or inhibitors by cellular NA or other sialic acid-binding enzymes. Consequently, this might result in cellular toxicity, ineffectiveness, or short lifetime of these compounds *in vivo*. On the other hand, if a non-sialate inhibitor that targets a non-substrate-binding site of the viral NA can be identified, it might offer a better alternative for treatment. To find a lead to test this strategy, we searched our chemical collection through a high-throughput screening assay. Interestingly, a well-known tuberculostatic, 4′-formylacetanilide thiosemi-

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carbazone (thiacetazone) (Colston et al., 1978), was identified. In this paper, we report a mechanistic study showing that thiacetazone targets the influenza A/WSN/33 NA at a site that is not the sialate substrate-binding pocket, but might be close to the calcium-binding domain of the enzyme. Since the calcium-binding site is essential for viral enzyme activity, and since this domain does not resemble the well-known calcium-binding EF-hand structure found in many cellular proteins (Persechini et al., 1989; Weinman, 1991), thiacetazone-type inhibitors might provide better selectivity between viral and cellular enzymes.

MATERIALS AND METHODS

Materials. 2-(3-Methoxyphenyl)-*N*-acetyl-D-neuraminic acid (MPN), salmonella and vibrio cholerae neuraminidases, and dideoxyacetylneuraminic acid were purchased from Sigma Chemical Co. 4-Methylumbelliferyl-α-oxo-*N*-acetylneuraminic acid was from Boehringer Mannheim. Thiacetazone was from Aldrich Chemical Co. or Sigma. The diazonium salt of 4-amino-2,5-dimethoxy-4′-nitroazobenzene (Fast black K salt) was also purchased from Aldrich. Cell culture media were obtained from Gibco Labs. $^{45}CaCl_2$ was from New England Nuclear/DuPont Co. Bio-Gel P-30 polyacrylamide columns were from Bio-Rad. All other chemicals were reagent grade. Pronase was purchased from Calbiochem. Influenza A N_1 virus WSN/33 was kindly provided by Matt Bui and Dr. Ari Helenius of Yale University (New Haven, CT) and by Dr. Doris Bucher of New York Medical College. Influenza A N_2 virus (NWS/Tokyo) was the generous gift of Dr. Yoshihiro Kawaoka, St. Jude Children's Hospital (Memphis, TN). The N_9 virus (A/NWS/G70C) and the type B virus (BHK/HG) were kindly supplied by Dr. Gillian Air, University of Alabama (Birmingham, AL). PR-8 virus was generously provided by Dr. Graeme Laver, The John Curtin School of Medical Research (Australia).

Purification of Influenza A/WSN/33 Virus. Influenza A/WSN/33 virus used for the preparation of neuraminidase was replicated in MDCK cells. Confluent cell monolayers in R-1500 roller bottles were usually inoculated with the virus at an infectivity of about 0.001 PFU/cell. Twenty minutes after the inoculation, cultures were treated with 4 μg/mL trypsin and incubated at 37 °C for 42 h. Virus titer was usually found to be 2×10^8 PFU/mL in the culture fluid at the end of incubation. The fluid was clarified with low-speed centrifugation and further purified with a sucrose step gradient. The purified virus was free of cellular protein as analyzed by SDS−PAGE and monitored by silver staining. The virus was stored in a storage buffer solution (10 mM Tris-HCl, 20% glycerol, and 1 mM DTT, pH 7.7) at −196 °C.

Purification of Neuraminidase. Influenza A/WSN/33 neuraminidase was obtained from the virus grown in MDCK cell culture. The structural stability and enzymatic activity of A/WSN/33 N_1 neuraminidase are highly dependent on the presence of Ca^{2+}. The purified enzyme denatured irreversibly in the absence or in the presence of low concentrations of Ca^{2+}. To purify the enzyme with good yield and high specific activity, methodology was developed specifically for A/WSN/33 neuraminidase. The purification procedure was adopted from that published by McKimm-Breschkin et al. (1991), with slight modifications for this particular strain. In a typical preparation of the neuraminidase extramembrane domain, 1 mL of 10 mg/mL A/WSN/33 virus particles in a Tris−saline buffer (20 mM Tris-HCl, 4 mM KCl, and 140 mM NaCl, pH 7.8) was treated with 100 μg of pronase so that the ratio of virus/pronase = 100/1 (w/w). The resultant suspension was incubated in an Eppendorf thermomixer at 35 °C for 65 min with constant shaking. The digested suspension was immediately centrifuged with an air-driven ultracentrifuge at 32 psi for 15 min. Supernatant containing soluble proteins was separated from the pellet that contained the NA-depleted virus particles and then centrifugally gel-filtered through a Bio-Gel P-30 polyacrylamide column that had been preequilibrated with buffer A (glycerol 25% (v/v), 20 mM K_2HPO_4, 50 mM KCl, 4 mM $MgCl_2$, and 5 mM $CaCl_2$, pH 5.85). The gel filtration allowed the pronase and short peptides, which were derived from the degradation of hemagglutinin during treatment, to be removed. The NA enzymatic activity recovered from this purification scheme was typically 95−120% of the original activity associated with the virions. Table 1 summarizes the yield and the enzyme specific activity at each purification step. The purified product was found to be nearly homogeneous as analyzed by SDS−PAGE and monitored by Coomassie Blue staining. The NA heads prepared by this procedure could be stored in liquid N_2 for at least 3 months without any significant loss of enzyme activity.

Table 1: Purification Table for the Neuraminidase from Influenza A/WSN/33 Virus

	protein mass (mg)	specific activity (μmol/mg/min)	purification (−fold)	yield (%)
Concentrated virus	10.3	0.28	1	100
pronase treatment	10.1	0.34		124
airfuged supernatant	0.25	11.7	42	113
Bio-Gel P-30	0.085	25.8	93	97

Neuraminidase Assay. The neuraminidase activity of virus particles or of the purified tetramer heads was assayed by utilizing an artificial substrate, 2-(3-methoxyphenyl)-*N*-acetyl-D-neuraminic acid (MPN), as described by Sedmak and Grossberg (1981). In our standard procedure, the enzyme was assayed in 60 μL of buffer B containing 20 mM NaH_2PO_4, 50 mM KCl, 4 mM $MgCl_2$, 10 μM $CaCl_2$, 0.2 mg/mL BSA, and 0.6 mM MPN, pH 5.85. The calcium was sometimes raised to higher concentrations when specified. The assay was run for 60 min at 32 °C, followed by the addition of 60 μL of buffer B (minus BSA) containing 3 mg/mL Fast black K salt (4-amino-2,5-dimethoxy-4′-nitroazobenzene) to convert the hydrolysis product, 3-methoxyphenol, to a diazonium complex as described (Sedmak & Grossberg, 1981). The purified enzyme or the virus used for the assay was usually adjusted to a concentration that gave an OD_{580nm} between 0.1 and 0.5, such that Beer's law could be applied. The extinction coefficient of the diazonium adduct determined under these experimental conditions was 19 530 cm^{-1} M^{-1}, as calibrated from a titration with 3-methoxyphenol.

A neuraminidase assay using the fluorescent substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MBN) was conducted as described by Potier et al. (1979). The assay was performed at 32 °C in a cuvette containing 500 μL of buffer B in which the substrate MPN was substituted

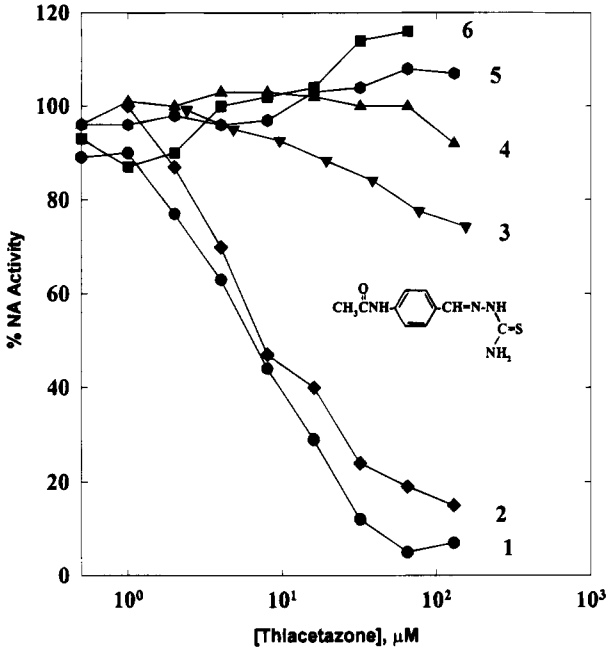

FIGURE 1: Specificity of thiacetazone inhibition. Neuraminidase activity was assayed in a 60 μL solution containing 5×10^{-3} unit of the designated NA and 10 μM Ca^{2+} as described in Materials and Methods. Curves: 1, influenza A/WSN/33 purified NA tetramer heads; 2, influenza A/WSN/33 virus particle containing the NA holoenzyme; 3, influenza A/PR-8 virus particle; 4, influenza A/NWS/Tokyo virus; 5, *Salmonella typhimurium* NA; 6, *Vibrio cholerae* NA. The inhibitor thiacetazone concentration varied from 0.5 to 130 μM as indicated. The chemical identity of thiacetazone is shown.

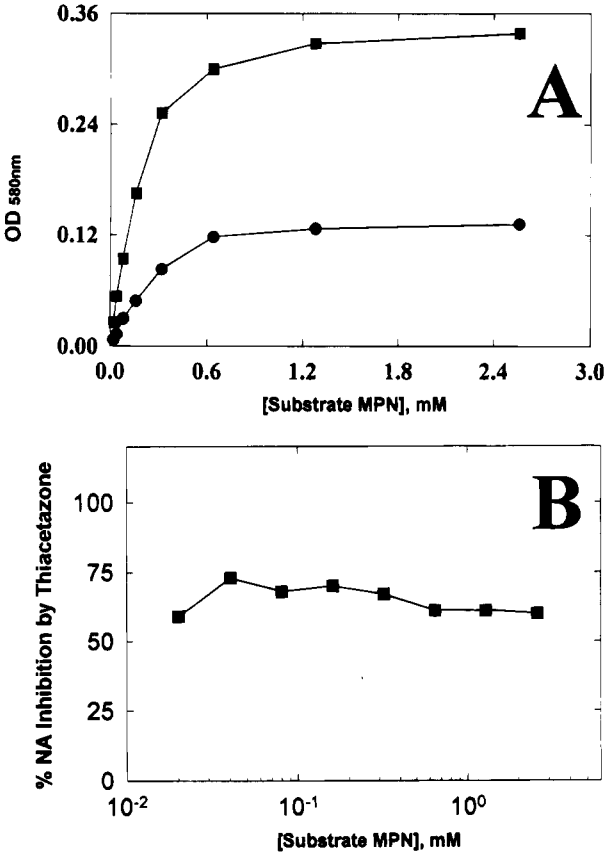

FIGURE 2: Effect of the substrate MPN on the inhibition of influenza A/WSN/33 NA by thiacetazone. (A) The NA activity of influenza A/WSN/33 virus was measured at various MPN concentrations, i.e., 20 μM to 2.56 mM, with (●) or without (■) 71 μM thiacetazone. (B) The fraction of inhibition was calculated from the NA activities with and without the inhibitor thiacetazone at each designated substrate concentration. The assay conditions were as described in Materials and Methods, except that the substrate MPN concentration was varied and $[Ca^{2+}] = 20$ μM.

by 0.2 mM MBN. The fluorescence at 450 nm (excited at 345 nm) was followed by a Perkin-Elmer fluorescence spectrophotometer (Model 650-10S). The slope of the fluorescence change at 450 nm vs time was used to calculate enzyme activity. Conditions were adjusted so that the slope was proportional to the quantity of the purified NA or the virus added to the assay mixture.

Plaque Assay. Madin–Darby canine kidney (MDCK) cells were acquired from the American Type Culture Collection (ATCC No. CCL 34, MDCK NBL-2) and routinely passaged in Eagle's Minimal Essential medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, and penicillin–streptomycin. In preparation for viral inhibition experiments, 35 mm polystyrene tissue culture plates (Falcon, Becton Dickinson Labs) were seeded with approximately 9.5×10^5 cells per plate and incubated at 37 °C with 5% CO_2. Under these conditions, confluent monolayers were obtained within 2–3 days and were used for viral inhibition experiments within 4–7 days.

Influenza A/WSN/33 virus that was used for infection had been passaged in chicken eggs and stored in liquid N_2. Prior to inoculation of the MDCK monolayers, concentrated virus stocks were diluted in Dulbecco's phosphate-buffered saline (D-PBS) without calcium chloride or magnesium chloride, but containing 5 mg/mL BSA. The inhibition assays were conducted as described by Hayden et al. (1980). Thiacetazone in culture medium with trace DMSO (<0.8%) was usually administered to monolayers 10 min before inoculation. Cells in each plate were then infected with 150–200 PFU of the virus, such that the plaque population was statistically adequate. A concentration of 2 μg/mL trypsin was always present in the medium to facilitate virus infection. Under these conditions, plaques with 1 mm diameter were usually obtained after a 24 h incubation period, and within 46 h the plaques grew to 4 mm.

RESULTS

Inhibition Specificity. Figure 1 shows that thiacetazone was remarkably specific to the influenza A/WSN/33 neuraminidase. It inhibited both the holoenzyme associated with the virion and the purified NA extramembrane domains from strain A/WSN/33, with an $IC_{50} = 8–15$ μM. The compound, however, was virtually ineffective for the other strains of virus, including A/PR-8 (N_1), A/NWS/Tokyo (N_2), A/NWS/G70C (N_9), and B/BHK/HG. Two bacterial neuraminidases, i.e., salmonella NA and vibrio NA, were also found to be insensitive to thiacetazone.

Inhibition Mechanism. Since the NA crystallographic data obtained from various strains indicate that the active site of the enzyme is highly conserved (Varghese et al., 1983; Bossart-Whitaker et al., 1993; Burmeister et al., 1992; Baker et al., 1987), it is very difficult to explain the high degree of specificity of thiacetazone for strain A/WSN/33 NA without assuming that the compound is an allosteric inhibitor. Whether or not thiacetazone indeed binds to a non-substrate-binding site was examined by a steady state kinetic study. Figure 2 shows that the inhibition of NA by thiacetazone

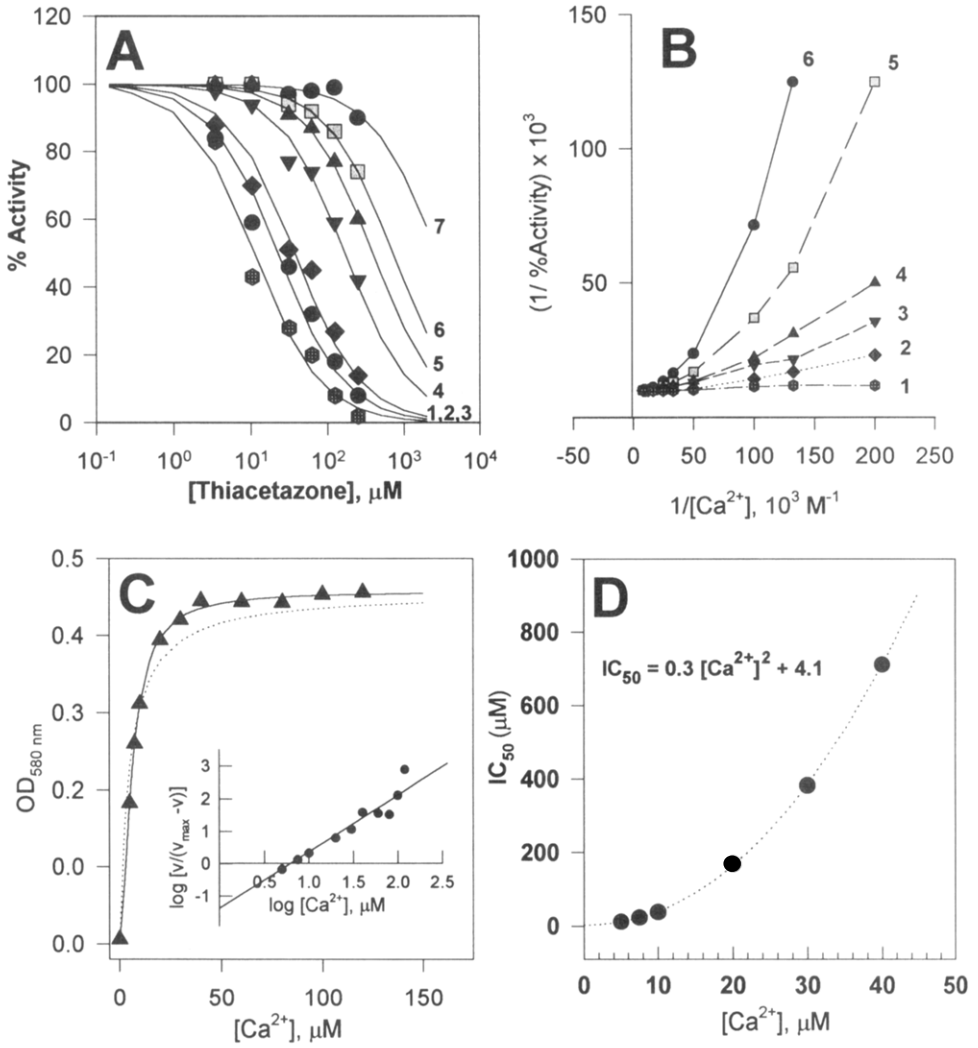

FIGURE 3: Protective effect of Ca^{2+} on the inhibition of influenza A/WSN/33 NA by thiacetazone. (A) The inhibition of influenza A/WSN/33 NA by thiacetazone was measured at various calcium concentrations: 5.0 (1), 7.5 (2), 10 (3), 20 (4), 30 (5), 40 (6), and 60 (7) μM. Assays were performed in duplicate, and the data were simulated by a nonlinear regression computer analysis based on the approximated equation, % inhibition = $100[I]/(IC_{50} + [I])$, where the free inhibitor concentration [I] was approximated by the total initial thiacetazone concentration. The symbols are experimental data and the curves are simulations. (B) The data in (A) were transformed and analyzed with a standard Lineweaver−Burk plot. Thiacetazone concentrations in each set of experiments were 3.5 (1), 10.4 (2), 31.3 (3), 63 (4), 125 (5), and 250 (6) μM. (C) Hill analysis for Ca^{2+} bound to the NA tetramer. The NA activity was measured at various calcium concentrations as described in Materials and Methods. The Hill coefficient n was determined by a nonlinear regression computer analysis based on the equation, $A = A_{max}[Ca^{2+}]^n/(K' + [Ca^{2+}]^n)$, where A and A_{max} represent the NA activities at nonsaturated and saturated calcium concentrations, respectively, and K' is $[Ca^{2+}]^n$ at which $A = 0.5A_{max}$. The triangles are experimental measurements, and the solid curve is the computer simulation that gave the best fit to the data. An n of 1.64 and a K' of 20.6 $\mu M^{1.6}$ were obtained from the simulation. As a comparison, a simulation based on the equation, $A = A_{max}[Ca^{2+}]/(K_d + [Ca^{2+}]$, where the site−site cooperativity was neglected (thus, $n = 1$), is shown as the dotted curve. The linear form of the Hill plot is shown in the inset, where $n = 1.6$ and $K' = 21$ were obtained. (D) IC_{50} of thiacetazone was plotted against $[Ca^{2+}]$. The dotted curve is the computer simulation that gave the best fit to the data based on eq 2 (see text). The K_i for thiacetazone bound to the neuraminidase was estimated to be 4 μM.

appeared to be independent of the substrate, 2-(3-methoxyphenyl)-*N*-acetyl-D-neuraminic acid, in the assay. Thiacetazone (71 μM) exhibited 70% inhibition of NA activity, and the inhibition remained unchanged while the MPN concentration varied from 20 μM to 2.56 mM.

In light of the observation by Kiyotani et al. that the calcium dependence of NA may be detected differently depending on which substrate was used (Kiyotani et al., 1987), an alternative assay utilizing a fluorescent substrate, 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (MBN), was also employed to verify inhibition by thiacetazone. The results from both assays qualitatively coincided (data not shown).

We have observed that the potency of thiacetazone was highly dependent on the calcium concentration in the medium. Figure 3A shows that thiacetazone was less inhibitory as the calcium concentration increased. The same data were transformed and analyzed with a Lineweaver−Burk plot, as shown in Figure 3B. The data in this figure demonstrate that thiacetazone and Ca^{2+} were mutually exclusive in binding to NA because the lines extrapolate to the same V_{max}. However, a repeatedly observed curvature of the plots indicates that the competition between thiacetazone and Ca^{2+} did not follow standard Michaelis−Menten kinetics and that the enzyme quite possibly contains multiple calcium-binding sites. As shown in Figure 3C, a Hill coefficient of 1.6 for Ca^{2+} bound to NA gave the best fit for the data, suggesting that the enzyme bound at least two calcium ions under the experimental conditions and that this multisite binding exhibited strong site−site cooperativity. The

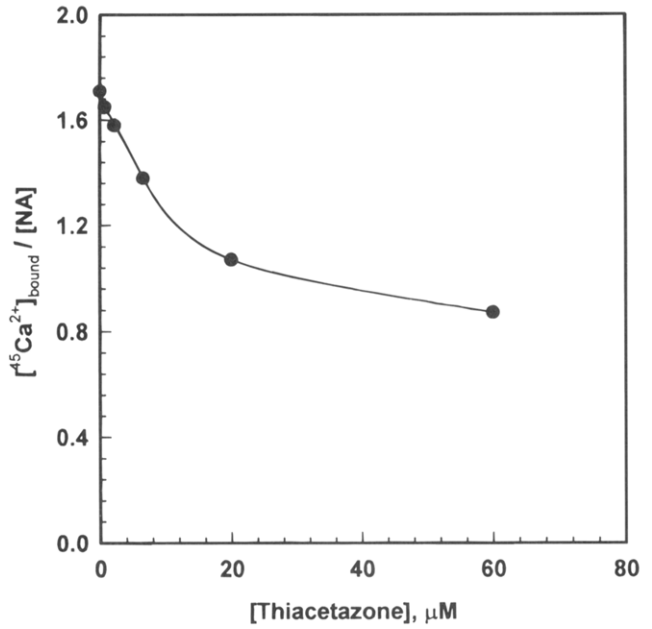

FIGURE 4: Substitution of NA-bound $^{45}Ca^{2+}$ with thiacetazone. Purified NA tetramer (5 μM) in buffer A containing 5 μM $^{40}CaCl_2$ was diluted 11-fold into the same buffer containing 5 μM $^{45}CaCl_2$ (final specific radioactivity = 80.5 μCi/mmol) and was subsequently allowed to equilibrate with 0, 0.75, 2.2, 6.6, 20, and 60 μM thiacetazone. One hundred and twenty microliters of each mixture was subsequently filtered through a 30-kDa cutoff membrane equipped in a pressure filtration chamber. The filtration was conducted under 20 psi of nitrogen and stopped when half the volume was through the membrane. The radioactivity in the lower chamber was counted to determine the free $^{45}Ca^{2+}$ concentration in the solution, whereas the cpm in the upper chamber was contributed by both NA-bound and free $^{45}Ca^{2+}$. The NA tetramer concentration in the upper chamber was determined by using Coomassie Blue. The stoichiometry of $[Ca^{2+}]_{bound}$/[NA] was calculated accordingly.

Hill dissociation constant K' obtained from this nonlinear regression was 21 $\mu M^{1.6}$.

For a competitive inhibitor, the inhibitory potency is a function of its competitor's concentration:

$$IC_{50} = ([S] + K_s)K_i/K_s \quad (1)$$

where IC_{50}, [S], K_i, and K_s are the half-inhibitory concentration, the competitor concentration, and the dissociation constants for the inhibitor and the competitor, respectively. Accordingly, if the substrate (competitor) binds to the enzyme at more than one site and the enzyme exhibits infinite cooperativity between sites, eq 2 can be obtained via the same algebraic operation used to derive the Hill equation:

$$IC_{50} = ([S]^n + K')K_i/K' \quad (2)$$

where n represents the number of competitor-binding sites, and K' is Hill dissociation constant. Empirically, since infinite ligand-binding cooperativity is physically impossible, n can only be taken as a nonintegral parameter related to the degree of cooperativity among interacting ligand-binding sites, rather than the number of the binding sites. Thus, similar to the Hill equation, eq 2 is utilized as an empirical curve-fitting guide to correlate the change in IC_{50} as the competitor concentration [S] varies. Figure 3D shows the correlation between thiacetazone IC_{50} and $[Ca^{2+}]$. The best fitting was obtained when $n = 1.95$ and $K' = 13$. Solution of eq 2 with $n = 1.95$ and $K' = 13$ gives $K_i = 4$ μM for thiacetazone bound to the neuraminidase.

If thiacetazone indeed binds to NA at a calcium-binding site, one would expect to detect the calcium released from the enzyme upon the addition of the compound. We used $^{45}Ca^{2+}$ to replace the endogenous $^{40}Ca^{2+}$ and then titrated the enzyme with thiacetazone. The results are shown in Figure 4. Under the indicated experimental conditions, 1.7 mol of $^{45}Ca^{2+}$ was initially substituted into each mole of NA tetramer and subsequently expelled by thiacetazone.

Effectiveness in MDCK Plaque Assay. Thiacetazone was tested *in vitro* for potential antiinfluenza activity with susceptible MDCK cell cultures infected by influenza A/WSN/33 virus. In Figure 5A−D, the virus was cultured for 24 h with or without thiacetazone. Both the number and the size of the plaques were effectively inhibited by the compound. The IC_{50} in this assay was estimated to be 250 μM, as measured by plaque counts in each plate. The effectiveness of thiacetazone in this assay seemed to be 1 order of magnitude less than that in the NA enzyme assay. This may be due to the presence of 1.8 mM $CaCl_2$ that was required in the culture medium. The compound in the same culture without the influenza virus showed no impediment to cellular viability as judged by a minimum cytopathogenic effect.

DISCUSSION

In the present work, we have demonstrated that thiacetazone, a non-sialate compound, inhibits both influenza A/WSN/33 neuraminidase activity and virus replication in an MDCK cell culture. The inhibition appeared to be highly specific since neither the enzymes from other strains of

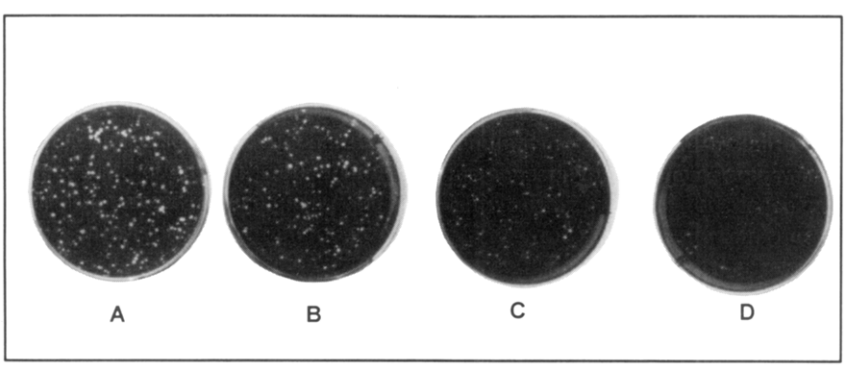

FIGURE 5: Protection of susceptible MDCK cells against influenza A/WSN/33 virus by thiacetazone: plaque formations in the presence of 0 (A), 100 (B), 400 (C), and 800 μM (D) thiacetazone. Cells in each culture were infected with 270 PFU of the virus. The conditions for growing the plaques were as described in Materials and Methods.

influenza virus nor bacterial NAs were affected by the compound. Apparently, thiacetazone distinguished between neuraminidases from different sources. This characteristic is distinct in comparison with traditional sialate-derivatized inhibitors, e.g., Neu5Ac2en, 4-amino-Neu5Ac2en, 4-guanidino-Neu5Ac2en, etc. (Meindl et al., 1974; Palese et al., 1974; von Itzstein et al., 1993). The sialate analogues are often effective across NAs from various strains of the virus and in some cases are even inhibitory against certain sialate-binding proteins, e.g., sialate aldolases (unpublished data). The unusual selectivity of thiacetazone prompted us to consider that this nonsialate inhibitor might inhibit the NA through a novel mechanism.

In our investigation, kinetic experiments showed that thiacetazone was mutually exclusive with the essential calcium ion, but unencumbered by the substrates MPN or MBN. This observation not only indicated the possible existence of an allosteric inhibitor site on the enzyme but also suggested that the site might be very close to the calcium-binding domain. The mutually exclusive kinetic behavior of thiacetazone and Ca^{2+} in binding to NA was not expected, since it was not anticipated that an organic compound was capable of competing with an inorganic cation for the same binding site on an enzyme. One interpretation for the observed competitive kinetics is that the inhibitor might bind to a site that is near the calcium-binding domain and subsequently induce a conformational change that expels the bound calcium ion, leading to the inactivation of NA. On the other hand, it is also possible that thiacetazone could be a calcium chelator that removes the essential calcium from NA. The latter seems to be unlikely, however, since the compound did not inhibit the other strains of influenza NA that were also calcium-dependent enzymes. Furthermore, when 2 mM thiacetazone was allowed to interact with 2 mM $CaCl_2$ in enzyme-free buffer, no chemical shift of the amide proton, as determined by NMR, was observed, thereby suggesting that there is no direct interaction between the compound and calcium ion (data not shown).

While the competition between thiacetazone and calcium provides an explanation for the inhibition mechanism, it is not immediately clear why the compound is effective for only a single strain of the virus. Comparisons of crystal structures and primary sequences of various influenza NAs have led Burmeister et al. to conclude that the calcium-binding sites, especially the one between the active site and a large surface antigenic loop, are conserved in all influenza A and B neuraminidases (Burmeister et al., 1992). Kinetic observations by Chong et al. also seem to be consistent with this contention (Chong et al., 1991). These observations may indicate that the thiacetazone-binding site is distinct from the calcium-binding site. The lack of inhibition of the other strains could be due to lack of the binding site or to thiacetazone's lack of the optimal configuration for binding, so that it only mildly affects A/WSN/33, a strain that binds Ca^{2+} relatively loosely, and fails to inhibit the others.

Although thiacetazone itself does not have the potency and efficacy across other strains of influenza virus to be therapeutically important, its mechanism of action might serve as a starting point to identify a compound that effectively modulates influenza NA through an allosteric pathway. Since a thiacetazone-type NA inhibitor is not a sialate analogue, the probability that it will interfere with, or be metabolized by, the other sialate-binding enzymes or proteins *in vivo* is low. Consequently, its likelihood of developing a mechanism-based toxicity should be diminished.

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