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Development of a Sensitive Chemiluminescent Neuraminidase Assay for the Determination of Influenza Virus Susceptibility to Zanamivir

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Determination of the sensitivity of influenza viruses to neuraminidase (NA) inhibitors is presently based on assays of NA function because, unlike available cell culture methods, the results of such assays are predictive of susceptibility *in vivo*. At present the most widely used substrate in assays of NA function is the fluorogenic reagent 2′-*O*-(4-methylumbelliferyl)-*N*-acetylneuraminic acid (MUN). A rapid assay with improved sensitivity is required because a proportion of clinical isolates has insufficient NA to be detectable in the current fluorogenic assay, and because some mutations associated with resistance to NA inhibitors reduce the activity of the enzyme. A chemiluminescence-based assay of NA activity has been developed that uses a 1,2-dioxetane derivative of sialic acid (NA-STAR) as the substrate. When compared with the fluorogenic assay, use of the NA-STAR substrate results in a 67-fold reduction in the limit of detection of the NA assay, from 200 pM (11 fmol) NA to 3 pM (0.16 fmol) NA. A panel of isolates from phase 2 clinical studies of zanamivir, which were undetectable in the fluorogenic assay, was tested for activity using the NA-STAR substrate. Of these 12 isolates with undetectable NA activity, 10 (83%) were found to have detectable NA activity using the NA-STAR substrate. A comparison of sensitivity to zanamivir of a panel of influenza A and B viruses using the two NA assay methods has been performed. IC_{50} values for zanamivir using the NA-STAR were in the range 1.0–7.5 nM and those for the fluorogenic assay in the range 1.0–5.7 nM (n = 6). The NA-STAR assay is a highly sensitive, rapid assay of influenza virus NA activity that is applicable to monitoring the susceptibility of influenza virus clinical isolates to NA inhibitors. © 2000 Academic Press

The sialic acid analogue zanamivir (4-guanidino-Neu5Ac2en) is a potent and selective inhibitor of influenza virus A and B NA[3] (sialidase, acylneuraminyl hydrolase, EC 3.2.1.18), preventing the release of progeny virions from infected cells and causing the formation of viral aggregates, thus limiting virus spread (1). The equipotency of zanamivir against influenza A and B virus NAs is in marked contrast to the only other antiviral agents used for the treatment and prevention of influenza virus infections, amantadine and its derivative rimantadine, which are active only against influenza A viruses. This narrow spectrum of activity, combined with poor side-effect profiles and the rapid development of drug-resistant strains, has severely limited the clinical use of both amantadine and rimantadine (2).

Variants of influenza A and B virus with reduced susceptibility to zanamivir have been isolated *in vitro* (3–8). However, studies by Gubareva *et al.* (3) have shown that the emergence of mutants of influenza that are resistant to zanamivir is a multistep process requiring prolonged exposure to inhibitor. Furthermore, mutations in more than one gene segment are frequently involved in the development of high levels of

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[3] Abbreviations used: NA, neuramidase; HA, hemagglutinin; NA-STAR, 1,2-dioxetane derivative of sialic acid; MDCK, Madin Darby canine kidney; MUN, 2′-*O*-(4-methylumbelliferyl)-*N*-acetylneuraminic acid; DMSO, dimethyl sulfoxide; Mes, 4-morpholineethanesulfonic acid.

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resistance *in vitro*, indicating that they are unlikely to occur rapidly in nature. To date, there has been only one case in which evidence for the emergence of resistance to zanamivir in a clinical setting has been obtained. In this case the prolonged administration of zanamivir to an immunocompromised patient resulted in the isolation of a virus containing mutations in both the NA and hemagglutinin (HA) genes after 12 days of drug administration. The NA of this mutant was shown to have a reduced level of sensitivity to zanamivir (9). Monitoring susceptibility of clinical isolates to zanamivir will therefore continue to be important as zanamivir, and other neuraminidase inhibitors, become more widely used.

Current assays used to determine the susceptibility of influenza virus clinical isolates to zanamivir include the plaque reduction assay, described by Hayden *et al.* (10), and the neuraminidase enzyme assay. During a phase II clinical trial (NAIB2008), Barnett *et al.* (11) carried out susceptibility monitoring using both enzyme neuraminidase assays and plaque reduction assays in Madin Darby canine kidney (MDCK) cells. They found that there was considerable variation in susceptibility to zanamivir in the plaque reduction assays. In addition, the results from this assay were found not to be predictive of sensitivity to zanamivir *in vivo* since isolates, which appeared to be insensitive in the plaque reduction assay, were highly susceptible to zanamivir in the ferret model. The greater precision of the fluorogenic NA assay utilizing 2′-*O*-(4-methylumbelliferyl)-*N*-acetylneuraminic acid (12) makes this assay more reliable for screening for changes that may confer clinical resistance to zanamivir that arises from mutation in the neuraminidase gene. In addition to being highly reproducible, the NA assays were also found to be predictive of *in vivo* susceptibility to zanamivir. However, in the same study, it was noted that several isolates possessed NA activity that was too low to be measured in the current fluorogenic NA enzyme assay, even after maximum concentration of the virus sample. As a result, there was no rapid method by which to assess the susceptibility of these isolates to zanamivir, and their drug sensitivity could only be established in a costly, time-consuming, and low-throughput *in vivo* assay. The purpose of this present study was to develop a new, more sensitive NA assay to allow susceptibility monitoring of viruses with low NA activity.

A chemiluminescence assay is described utilizing a new 1,2-dioxetane derivative of sialic acid (NA-STAR). The dioxetanes have emerged as the most important chemiluminogenic substrates and are described by Bronstein *et al.* (13) and Edwards *et al.* (14). The advantages of chemiluminometry in comparison to fluorometry are discussed and the sensitivities of the two assays are compared.

MATERIALS AND METHODS

Materials

NA was purified from B/Beijing/1/87 which had been grown in embryonated hens' eggs (Solvay Duphar, Netherlands) using established methods (15). Clinical isolates of influenza virus from the zanamivir phase II clinical study (NAIB2008) were passaged once in MDCK cells. A panel of influenza A and B viruses was also passaged once in MDCK cells and obtained from the National Institute for Medical Research (Mill Hill, London, UK).

2′-*O*-(4-Methylumbelliferyl)-*N*-acetylneuraminic acid (MUN) was obtained from the Sigma Chemical Company (Poole, Dorset, UK). NA-STAR 1,2-dioxetane chemiluminescent substrate was custom synthesised by Tropix Inc. (Bedford, MA). Light Emission Accelerator II–10% Sapphire II enhancer in 0.1% diethanol amine, pH 10 (hereby referred to as Sapphire enhancer) and the white Costar microtiter plates were also provided by Tropix Inc. Black microfluor 96-well plates were obtained from Dynatech Laboratories Inc. (Chantilly, VA) and white 96-well Packard Optiplates were obtained from the Packard Bioscience Company (Meriden, CT). Zanamivir was synthesized at Glaxo Wellcome Medicines Research Centre (Stevenage, UK). All other chemicals and reagents were obtained from the Sigma Chemical Company (Poole, Dorset, UK).

Methods

*Synthesis of the Chemiluminescent Substrate **6** as Described by the Scheme (Fig. 1)*

*Methyl (5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-β-D-galactononulopyranosyl chloride)onate **3.*** The chloride **3** was prepared in two steps from the commercially available *N*-acetylneuraminic acid **1**, according to the literature procedure (16). The crude chloride **3** was purified by a silica gel plug, eluting with 200 ml of 80–90% EtOAc in hexanes. After concentration of the filtrate, the chloride **3** was obtained as an off-white powder and was used immediately for the next coupling reaction.

*Methyl (2-chloro-5-(methoxy-5-chlorotricyclo[3.3.1.1^{3,7}]-dec-2-ylidenemethyl)phenyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosid)onate **4.*** 2-Chloro-5-(methoxy-5-chlorotricyclo-[3.3.1.1^{3,7}]dec-2-ylidenemethyl)phenol **7** (1.65 g, 4.86 mmol (Fig. 2)) and the phase transfer catalyst tetrabutylammonium hydrogen sulfate (0.83 g, 2.43 mmol) were placed in a 100-ml round-bottomed flask and treated with 12.5 ml of CH_2Cl_2 and 17.5 ml of 0.5 N NaOH at room temperature. To the resulting two-phase mixture a solution of the chloride **3** (1.24 g, 2.43 mmol) in 5 ml of CH_2Cl_2 in one portion was added. After an hour of vigorous stirring, the reaction mixture

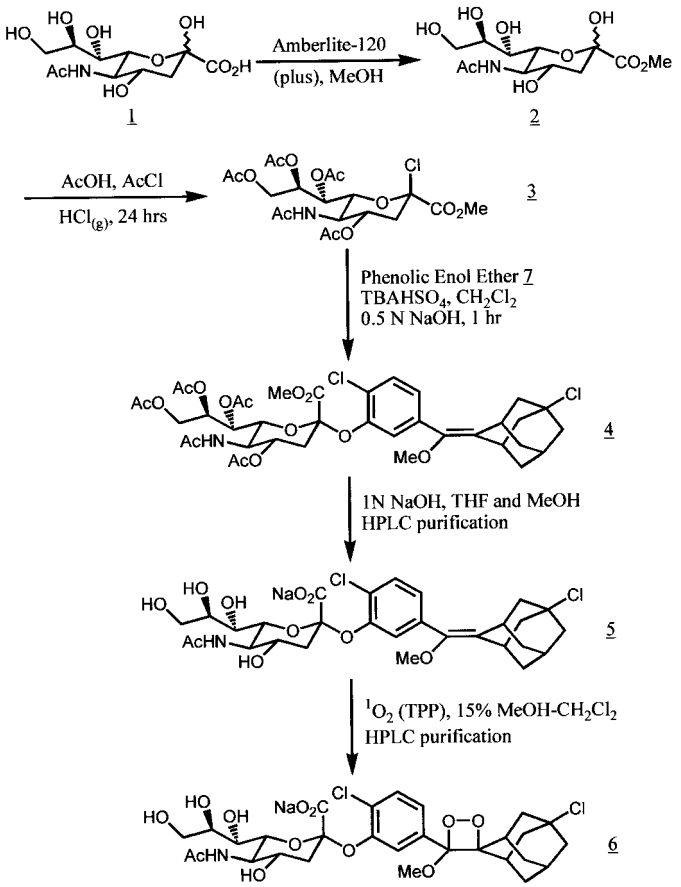

FIG. 1. Synthesis of NA-STAR 1,2-dioxetane substrate.

was diluted with CH_2Cl_2 and poured into a separatory funnel, containing saturated sodium bicarbonate solution. After the organic layer was separated, the aqueous layer was further extracted two more times with CH_2Cl_2. The combined organic layers were washed with H_2O and dried over anhydrous Na_2SO_4. TLC (80% EtOAc in hexanes) showed the coupling product **4** at R_f = 0.48 with faint shadows above and below. The organic solution was treated with 10 drops of Et_3N and concentrated.

The crude product was purified by the silica gel chromatography, eluting with 20% EtOAc in hexanes to recover the unreacted enol ether phenol **7**, followed by 80–90% EtOAc in hexanes, affording 1.242 g (62.9%) of the product **4** as a light yellow, crispy foam. IR ($CHCl_3$, cm^{-1}): 3432, 3040, 2936, 1750, 1688, 1372, 1235, and 1040. The ^1H NMR ($CDCl_3$) spectrum was complicated, but it still revealed that the sample actually was a mixture of about 4.3:1 of the desired product **4** and glycal from the dehydrochlorination of the chloride **3**. A clean sample was obtained by the process of removing the *O*-acetyl groups with NaOMe in MeOH, and reacylation with acetic anhydride in pyridine to remove the glycal. The resulting ^1H NMR spectrum clearly showed the product **4** existed as a 1:1 mixture of two isomers, based on the equal splitting of the methyl ester and *O*- and *N*-acetyl methyl signals. The presence of two triplets at 2.82 and 2.86 ppm for H-3e of the neuraminic acid ring indicated that both of them are the α-pyranosides.

^1H NMR ($CDCl_3$): δ7.35 (d, J = 8.2 Hz, 1H), 7.21 (m, 1H), 6.99 (m, 1H), 5.25–5.34 (m, 3H), 4.98–5.10 (m, 1H), 4.14–4.31 (m, 3H), 4.03 (m, 1H), 3.75 and 3.747 (2s, 3H, Me ester), 3.43 (broad s, 1H), 3.30 (s, 3H, OMe), 2.86 and 2.82 (2t, J = 4.3 Hz, 1H), 2.13, 2.12, 2.10, and 2.07 (4s, 6H), 2.04 (s, 3H), 2.03 (s, 3H), 1.91 (s, 3H), and 1.67–2.29 (m, 12H, 11 adamantyl H and H-3a).

Sodium (2-chloro-5-(methoxy-5-chlorotricyclo[3.3.1.1^{3,7}]-dec-2-ylidenemethyl)phenyl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosid)onate ***5***. The impure pyranoside **4** (1.76 g, 2.1 mmol) was deprotected in a mixture of 6.5 ml each of THF and MeOH with 12 ml of 1 N NaOH at 0°C. After 5 min at 0°C, the mixture was stirred at room temperature for an hour. The pH was lowered by the addition of solid sodium bicarbonate (1.05 g). Most of the bicarbonate did not go into solution; however, a clear solution was ultimately obtained by dilution with water yielding a total volume of 100 ml. The solution was filtered on a Buchner funnel, rinsed with small volume of water, and purified by reverse-phase prep HPLC with a 1-in. column packed with polystyrene. The column was eluted with an acetonitrile–water gradient. The fractions containing the product were pooled and lyophilized, to yield 659 mg (46.6%) of **5** as a white fluffy powder.

^1H NMR (D_2O): δ7.42 (d, J = 8.1 Hz, 1H), 7.32 (broad s, 1H), 7.02 (d, J = 8.1 Hz, 1H), 3.72–3.93 (m, 5H), 3.59–3.68 (m, 2H), 3.31 (s, 1H), 3.31 (s, 3H, OCH_3), 2.90–2.99 (m, 1H, H-3e), 2.67 (broad s, 1H), 2.08–2.30 (m, 6H, adamantyl), 2.02 (s, 3H, N-Ac), 1.66–2.0 (m, 5 adamantyl H and 1-H-3a).

Sodium (2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2′-(5-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl-phenyl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosid)onate ***6***. Photooxygenation of a solution of **5** (414.5 mg, 0.635 mmol) in 20 ml of 15% MeOH in CH_2Cl_2 in the presence of 20 drops of tetraphenyl-

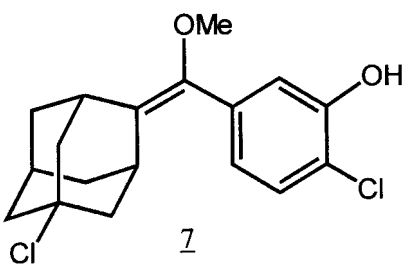

FIG. 2. Phenolic enol ether.

porphine stock solution (2 mg/ml in $CHCl_3$) was carried out by irradiation with a 400 W sodium vapour lamp while continuously bubbling oxygen through the solution for 25 min at 3–5°C. The reaction was monitored using the UV spectrum: the maximum absorption of the product shifted from 260.5 nm to 277.5 nm as the reaction proceeded. The mixture was concentrated on a rotary evaporator at low temperature, followed by pumping *in vacuo* until a purple, glassy foam was obtained. The crude product was soluble in 30 ml of water containing 2 ml of saturated $NaHCO_3$ solution. Filtration through a Buchner funnel and rinsing with water yielded a final volume of 50 ml. The solution was injected in five portions on the reverse-phase HPLC column described above. The column was eluted with an acetonitrile–water gradient. It was found that a broad peak eluted just before the sharp major peak. Both product fractions exhibited chemiluminescence upon treatment with neuraminidase enzyme (recombinant from *Salmonella typhimurium*) obtained from Oxford Glycosciences. These fractions were combined and lyophilized to yield 418.4 mg of white, fluffy 1,2-dioxetane product as a mixture of isomers.

Fluorescence Assay for NA Using MUN as Substrate

The NA assay using MUN as substrate was based on the method described by Woods *et al.* (17), with the exception that water was used in place of the DMSO for the inhibitor dilutions and the concentration of MUN used was 65 μM. This concentration had previously been determined as the K_m concentration for the enzyme using MUN as substrate. Initially, the purified NA was titrated by serial twofold dilutions in 32.5 mM Mes, pH 6.5, and 4 mM $CaCl_2$ and the concentration of NA against NA activity was plotted graphically to determine the enzyme concentration to be used in subsequent inhibition assays. An exact concentration of the enzyme stock was determined by using zanamivir as an active site titrant. For experiments using the clinical isolates, the samples were assayed undiluted. In fluorescence, the responses obtained for the samples (signal, S) and the blanks (background, N), expressed in arbitrary units (relative fluorescence units, RFUs), were divided to yield signal-to-noise ratios (S/N). 4-Methylumbelliferone was quantified by fluorometric determination using a Wallac Victor 2 multilabel counter (hereby referred to as Wallac counter) with an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Wells were read at a rate of 1.0 s per well. The data were plotted as log zanamivir concentration against percentage control and the IC_{50} values were determined using Grafit 3.09. The results were fitted using a four-parameter logistic and IC_{50} values were determined using the equation, %control = $(v_0/1 + ([I]/IC_{50})^s)$ + background, where s is the slope factor, $[I]$ is the inhibitor concentration, and v_0 is the maximum y range.

Chemiluminescence Assay for NA Using NA-STAR as Substrate

Influenza virus NA was initially titrated by serial twofold dilutions in 32.5 mM Mes, pH 6.0, and 4 mM $CaCl_2$. The concentration of NA against S/N was plotted graphically to determine the enzyme concentration to be used in subsequent inhibition assays. An exact concentration of the enzyme stock was determined by using zanamivir as an active-site titrant. For experiments using the clinical isolates, the samples were assayed undiluted. For zanamivir inhibition assays, a stock solution of zanamivir (3 mM) was prepared in water and then serially diluted from 0.55 μM to 0.028 nM in water. A volume of enzyme (40 μl), diluted in 32.5 mM Mes, pH 6.0, and 4 mM $CaCl_2$, was preincubated $\pm$10 μl zanamivir for 30 min at room temperature in white 96-well Packard Optiplates. The reaction was initiated by the addition of 5 μl of a 1:15 dilution of NA-STAR substrate prepared in 32.5 mM Mes, pH 6.0, and 4 mM $CaCl_2$. The final concentration of substrate in the assay was 60 μM. This concentration had previously been determined as the K_m concentration for the enzyme using NA-STAR substrate. Fresh stock solutions of NA-STAR (10 mM) were prepared in 0.51-M sodium acetate buffer, pH 8.3, on a daily basis. The reaction mixture was then incubated at 37°C for 15 min with shaking. After the enzymatic cleavage of NA-STAR, chemiluminescent light emission was triggered by adding 55 μl of the Sapphire enhancer to the assay mixture in each well. The chemiluminescence was read immediately in a Wallac counter (refer to results and discussion section). A protocol with a luminescence label, no excitation, no filter, and a counting time of 0.1 s per well was utilized. The data were plotted as log zanamivir concentration against percentage control (where control consisted of enzyme and substrate with water in place of zanamivir) and the IC_{50} values were determined using Grafit 3.09. The results were fitted using a four-parameter logistic and IC_{50} values were determined using the equation, %control = $(v_0/1 + ([I]/IC_{50})^s)$ + background, where s is the slope factor, $[I]$ is the inhibitor concentration, and v_0 is the maximum y range. Where signal to noise values were obtained for the undiluted clinical isolates, photon emissions were quantified in either a Wallac counter or a Tropix TR717 luminometer (hereby referred to as Tropix luminometer).

RESULTS AND DISCUSSION

Chemiluminescence is a highly sensitive technique for monitoring enzyme activity due to low background

luminescence coupled with high-intensity light output due to enzyme catalysis. In addition, the 1,2-dioxetane chemiluminescent substrates in particular are nonradioisotopic and provide a high-intensity signal with low background and a wide dynamic range. The benefits of increased detection sensitivity provided by chemiluminescent 1,2-dioxetane enzyme substrates compared to fluorometric substrates have been widely demonstrated. AMPGD, a phenylgalactose-substituted 1,2-dioxetane chemiluminescent substrate, has been described by Bronstein *et al.* (18), and has been used in connection with reporter gene assays. Jain *et al.* (19) studied the utility of AMPGD substrate for the detection and quantification of β-galactosidase. They were able to detect as little as 2 fg of β-galactosidase compared to 200 fg, which was detected in a fluorescence-based assay. This increase in sensitivity is comparable to that obtained in the present study. Van Poucke *et al.* (20) also utilized this 1,2-dioxetane derivative as a substrate and found that it afforded severalfold increases in sensitivity compared to fluorescent techniques.

Examples of other 1,2-dioxetane substrates include the Galacton-STAR substrate also for β-galactosidase. Martin *et al.* (21) showed that with this substrate as little as 10 fg of purified enzyme could be detected at a S/N ratio of 2, and with a dynamic range of 5 to 6 orders of magnitude of enzyme concentration. Both Galacton-STAR and NA-STAR are versatile reagents for highly sensitive, fast, and simple detection that offer more sensitive alternatives to current methodologies. Bronstein *et al.* (22) have also demonstrated the use of 1,2-dioxetane substrates in the detection of β-galactosidase (Galacton and Galacton-plus), β-glucuronidase (Glucuron), and secreted placental alkaline phosphatase (CSPD). In agreement with the findings in this study, chemiluminometry afforded increased sensitivity over several orders of magnitude compared to that provided by fluorescence-based assays. Use of all substrates for the three enzymes resulted in a significantly advanced level of detection compared with fluorescence-based assays. When considering the design of a more sensitive neuraminidase assay, we therefore chose to synthesize dioxetane sialosides as potential chemiluminescent substrates of NA.

To assess the potential of NA-STAR to detect levels of neuraminidase activity that are below the level of quantification of the fluorogenic assay using MUN, the limits of detection were compared in assays run under identical conditions. The detection threshold of the chemiluminescence NA assay using NA-STAR as substrate was 3 pM (Fig. 3). This was at least 67-fold greater than the sensitivity provided by the current MUN fluorometric assay, with which a concentration of NA no less than 200 pM could be detected. The detection threshold is the minimally required difference between the signal and the background (noise) which indicates a positive response and determines the ability of an assay to detect small concentrations of analyte. Although we chose to take a S/N ratio of 2 as the limit of detection of the assay, plots of S/N against enzyme concentration for the two assays have similar slopes, indicating the difference in the sensitivity of the two assays is independent of the S/N ratio used in the comparison.

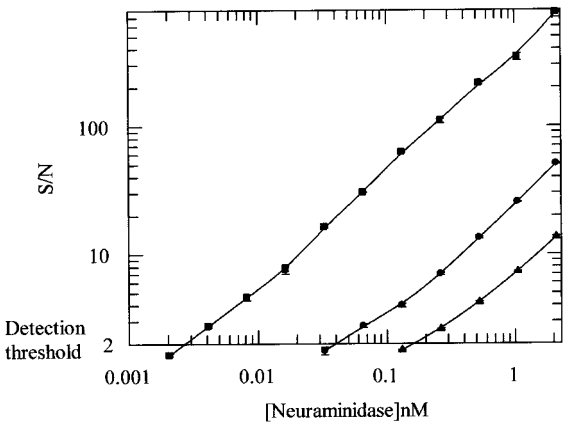

FIG. 3. Comparison of the detection threshold of the fluorescence and chemiluminescence assay. The ratio of signal to noise at different concentrations of NA (indicated by the *x* axis) was determined using NA-STAR (● and ■) or MUN (▲) as substrate. (1.0 nM NA is equivalent to 0.0028 μg of NA/55 μl assay volume, based on a subunit molecular weight of 50,000). NA was diluted prior to assay in 4 mM $CaCl_2$ in combination with either 32.5 mM Mes, pH 6.5, for the fluorescence assay or 32.5 mM Mes, pH 6.0, for the chemiluminescence assay. The NA-STAR and MUN assays were conducted as outlined under Materials and Methods. Chemiluminescent light emission was triggered by adding either Sapphire enhancer, pH 10 (■), or 500 mM glycine buffer, pH 10 (●). The data displayed are the mean and where errors are large enough to be displayed, these represent the mean ± SE (n = 3).

Optimal detection sensitivity in the chemiluminescence assay was obtained when the reaction was terminated with the Sapphire enhancer. Use of the Sapphire enhancer yielded a chemiluminescent signal that was ~100 times greater than that obtained with a pH 10 glycine buffer and, although the background signal also increased, this increase was less pronounced so that the S/N ratio increased by a factor of 5. The use of an enhancement solution such as the Sapphire enhancer reduces the water-induced quenching effect that can occur. It functions to separate the water from the site of chemiluminescence signal production, creating a hydrophobic environment, which increases the quantum efficiency of light production, and produces an intense light signal. Figure 3 also shows that a significant change in the detectability of the chemiluminescence assay was observed when the enhancer was used. The Sapphire enhancer was used in all the experiments carried out in this study. A variety of

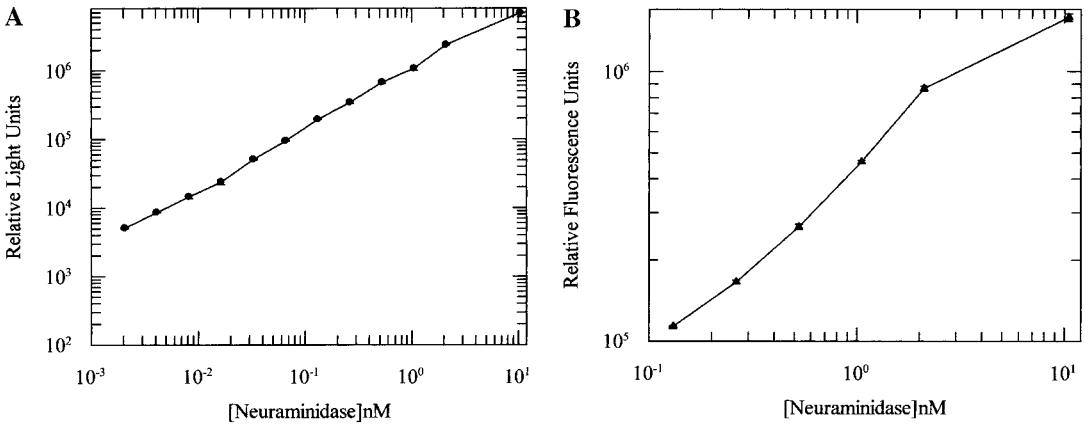

FIG. 4. Graphs showing the dose–response curves generated by different substrates for neuraminidase. The chemiluminescent NA-STAR assay (A) and the fluorometric MUN assay (B) were conducted as described under Materials and Methods. NA was diluted prior to assay in 4 mM $CaCl_2$ in combination with either 32.5 mM Mes, pH 6.5, for the fluorescence assay or 32.5 mM Mes, pH 6.0, for the chemiluminescence assay. The concentrations of NA used are indicated on the *x* axis. The data displayed are the mean and where error bars are large enough to be displayed, these represent the mean ± SE (n = 3).

different enhancement solutions is available and the choice of enhancement solution depends on the particular requirements of an assay. A study by Jain *et al.* (19) was carried out with the use of Emerald enhancer. Conversely to the findings in this study, they found no significant change in the detectability of the assay using the enhancer. This may be because although the signal intensity is higher using the Emerald enhancer, the background noise is also higher.

In addition to an increase in the sensitivity of the NA assay, the chemiluminescent assay also offered an increased dynamic range over that of the fluorescence assay. This allowed the potential for a more accurate discrimination between small differences in NA concentration to be made. At the detection threshold, the chemiluminescent signal/photon count obtained was approximately 7000 at an enzyme concentration of 0.003 nM, rising to counts of $\sim 7 \times 10^6$ at an enzyme concentration of 10.5 nM, which was the maximum enzyme concentration used in this study. In this study, the chemiluminescent signal therefore ranged over four orders of magnitude (Fig. 4A). In contrast, at the detection threshold of the fluorescence assay, the counts obtained were approximately 1×10^5 fluorescence units at an enzyme concentration of 0.2 nM, rising to counts of $\sim 1 \times 10^6$ at an enzyme concentration of 10.5 nM. For the enzyme concentrations used, the dynamic range (signal range) of the fluorescence assay was therefore no more than two orders of magnitude (Fig. 4B).

During the development of the chemiluminescent assay, a color-quenching effect was noted when assaying clinical samples. This was found to be due to the phenol red used as a pH indicator in the tissue culture medium present during the amplification of the virus stock. In an attempt to solve this problem the Emerald enhancer was used in place of the Sapphire enhancer because the Emerald enhancer causes light to be emitted in the green part of the spectrum, and hence was expected to reduce the colour quenching effect. However, no improvement was observed using this enhancer. To reduce the quenching effect, it therefore became necessary to reduce the final concentration of phenol red in the assay. A quench curve for phenol red was constructed (Fig. 5) and 2 mg/liter was established as the maximum tolerable concentration of phenol red in the assay. If it is necessary to remove any phenol red present in a sample prior to assay, this could be achieved by dialysis or gel filtration. However, effects

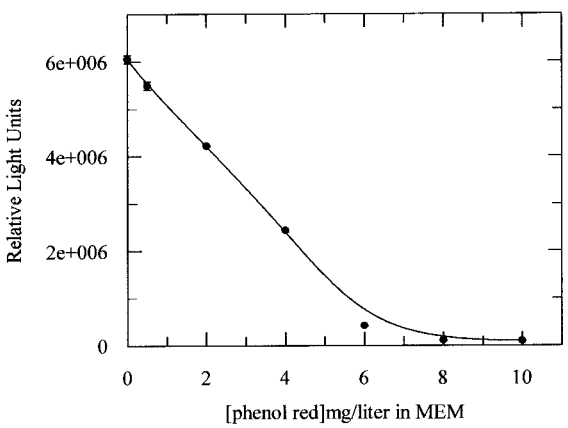

FIG. 5. Quench curve for phenol red. The NA-STAR assay was conducted as outlined under Materials and Methods. The NA was diluted in 32.5 mM Mes, pH 6.0, 4 mM $CaCl_2$ ± phenol red in minimum essential medium (MEM) prior to assay, such that the final concentration of NA in the assay was 10.5 nM. The final concentrations of phenol red in the assay were as indicated by the *x* axis. The data displayed are the mean and where errors are large enough to be displayed, these represent the mean ± SE (n = 3).

TABLE 1

Comparison of the Tropix Luminometer and the Wallac Counter—Signal to Noise and Cross-talk Performance

	Tropix luminometer		Wallac counter	
Plate type	S/N at 0.0021 nM NA	Crosstalk (%) at 1.05 nM NA	S/N at 0.0021 nM NA	Crosstalk (%) at 10.5 nM NA
White Packard Optiplates	10.7	0.009	1.2	0.05
White Costar plates (Tropix)	3.0	0.003	1.5	0.06
Black Dynatech plates	4.3	0.002	3.0	0.02

Note. The S/N ratios were obtained using all three plate types on both the Tropix luminometer and the Wallac counter using a concentration of NA at approximately the detection threshold of the assay (~2–3 pM). The cross-talk performance was measured at the highest possible enzyme concentration that could be used for each instrument without causing the generated data to be off-scale. This equated to 1.05 nM with the Tropix luminometer and 10.5 nM with the Wallac counter. The NA-STAR assay was carried out as outlined under Materials and Methods. The data displayed are the mean, with $n = 3$.

on stability and concentration of the sample could result in considerable loss of enzyme activity. This could be very detrimental to those samples in which there is little enzyme activity present in the first instance, such as those from the NAIB2008 clinical trial. We therefore recommend that phenol-red-free medium be used in the amplification of the virus stock.

An additional problem encountered during the development of the chemiluminescence assay was the effect of "cross-talk" when the assay was run in 96-well microtiter plates. Cross-talk is the leakage of light from one well into an adjacent well and can contribute a significant error in wells with low signal which are next to wells of higher signal. Cross-talk occurs via two distinct pathways, one being the instrumentation and the other the type of microplate. The cross-talk performance was evaluated for both the Wallac counter and the Tropix luminometer, and also for three different microtiter plates, black Dynatech plates, white Costar plates, and white Packard Optiplates (Table 1). Generally the cross-talk measured with the Tropix luminometer was 10-fold lower than that measured with the Wallac counter for all three plate types. The percentage cross talk measured was lowest for the black plates; however, this is due to the fact that these plates absorb a large amount of the emitted light, and hence the overall signal is also low. Black plates are generally regarded as poor performers for chemiluminescence-based assays (Tropix web page, http://38.248.78.65/717faq.htm). Figure 6 compares the counts obtained using black plates and white plates. The two different white plates gave similar cross-talk performance for all three plate types on both instruments. On the whole the Tropix luminometer performed better then the

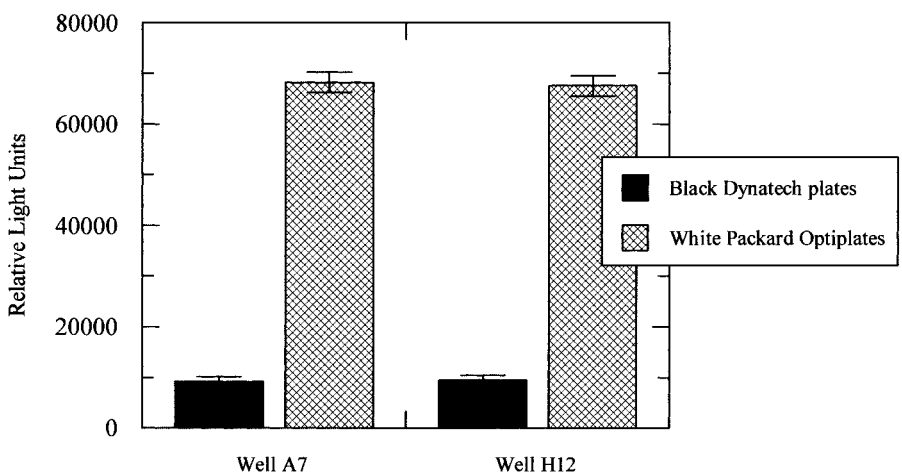

FIG. 6. A comparison of white plates and black plates for use in the NA-STAR chemiluminescent assay. The NA-STAR assay was conducted as outlined under Materials and Methods. The NA was diluted in 32.5 mM Mes, pH 6.0, 4 mM $CaCl_2$ prior to assay such that the final concentration in the assay was 10.5 nM. Identical samples of NA and NA-STAR substrate reaction mix were placed into wells A7 and H12 in both plate types. Chemiluminescent light emission was triggered by the addition of 500 mM glycine buffer, pH 10. The data displayed are the mean and where errors are large enough to be seen, these represent the mean ± SE.

Wallac counter, although it has the disadvantage that it can be used solely for luminescence applications. The Wallac counter is more economical in that it can be used for a variety of different assay technologies.

To assess the effect of the cross-talk on zanamivir IC_{50} determinations, an IC_{50} value was determined for a NA concentration with low signal (S/N = 2). The same determination was carried out a second time, but this time in close proximity on the microtiter plate to a NA concentration with high signal (S/N = 40, 80, and 160). No effect on the IC_{50} value of the enzyme at low S/N was observed. However, in all susceptibility assays, the level of input virus used influences the IC_{50} values determined. It was therefore decided to standardize the level of virus used in the chemiluminescence assay so that the S/N obtained was no more than 40/1. Controlling the amount of virus in this way reduces the level of variability that may occur in the assay with higher viral levels, avoids incorrect reporting of resistance, and also eliminates any cross-talk. Using the Wallac counter with white Packard Optiplates there is negligible cross-talk at a S/N of 40/1.

A proportion of the clinical isolates from the phase II clinical trial (NAIB2008) was found to have undetectable NA activity in the current fluorogenic assay. When tested in the chemiluminescence assay, NA activity could be detected in 83% of these isolates (Table 2). The S/N ratios were obtained with both the Tropix luminometer and the Wallac counter for the three plate types mentioned above. Little difference in the S/N ratios was observed for both instruments and all three plate types (Fig. 7). When the S/N ratios were obtained using the clinical isolates, the phenol red concentration exceeded 2 mg/liter, which had previously been chosen as the maximum concentration of phenol red that could be present in the assay without causing a significant decrease in the chemiluminescent signal. Since future susceptibility monitoring will be carried out at a level of phenol red that is acceptable (i.e., below 2 mg/liter), this allows the potential for the remaining 17% of the isolates also to be detected in the chemiluminescence assay.

For the optimization of the NA-STAR assay, the levels of calcium, as well as the pH and the length of incubation time were varied (data not shown). There was no change in the sensitivity of the assay when the concentration of calcium was varied from 4 to 20 mM. Calcium has been shown to increase the initial rate by accelerating the enzyme–substrate interaction (23). A concentration of 4 mM was chosen for all future experiments. The effect of the pH of the reaction buffer was studied by varying the pH between 5.5 and 6.5, since the pH optima of viral NAs lie in this range. Similarly, no increase in sensitivity was observed at differing pH. No effect on the chemiluminescent signal or the S/N was observed and a pH of 6.0 was chosen for all future experiments. To establish the optimal incubation time, the reactions were incubated at 5-min intervals up to 60 min. There was a steady increase in the chemilumi-

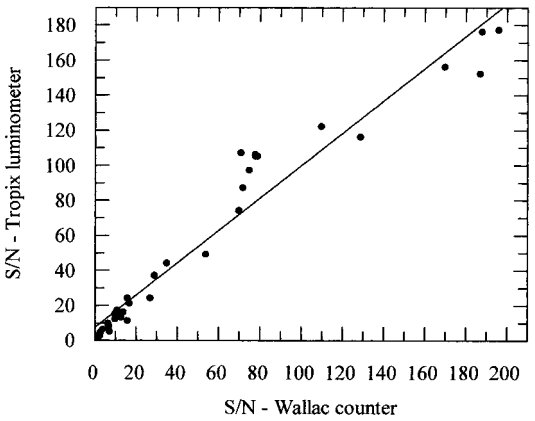

FIG. 7. A comparison of the signal to noise obtained with the Wallac counter and the Tropix luminometer. The ratio of signal to noise was obtained with undiluted clinical samples from the NAIB2008 trial using either the Tropix luminometer or the Wallac counter. Black Dynatech, Packard Optiplates, and white Costar plates were used. The NA-STAR assay was conducted as outlined under Materials and Methods. The data displayed are the mean (n = 2) and show that the S/N obtained was similar for both instruments.

TABLE 2

Comparison of the Fluorescence and the Chemiluminescence Assays Using Clinical Samples from the NAIB2008 Trial

Clinical samples from NAIB2008	Fluorescence assay (S/N)	Chemiluminescence assay (S/N)
7221	BQL	BQL
7221 (conc)	BQL	BQL
7222	BQL	BQL
7222 (conc)	BQL	BQL
7227	BQL	10
7230	BQL	BQL
7230 (conc)	BQL	1.4
7236	BQL	11
7238	BQL	35
7239	BQL	29
7241	BQL	2.4
7244	BQL	27
7245	BQL	11
7257	BQL	14
7260	BQL	17

Note. The clinical samples were assayed undiluted using either MUN or NA-STAR as substrate at a concentration of 100 μM. The NA-STAR and MUN assays were conducted as outlined under Materials and Methods except that the concentration of MUN and NA-STAR used was 100 μM. Where samples were concentrated (conc), this was achieved by centrifuging the samples in centriprep tubes with a 10,000 molecular weight cutoff point. In each case the S/N was obtained by dividing the signal by the background and where the signal obtained was too low to be quantified this was denoted by BQL, below quantifiable level.

nescent signal up to 15 min, where it peaked and then proceeded to gradually decrease. As a result of this observation, all future experiments were conducted using a 15-min incubation time. Also, after a period of 15 min, a gradual increase in the chemiluminescent signal in the blanks was observed, indicating that there was a possibility of nonenzymatic substrate hydrolysis occurring during longer incubation times.

Due to the relatively short-lived chemiluminescent signal produced upon termination of the reaction, (half-life of approximately 5 min, Fig. 8), it was important to read the plate immediately after stopping the reaction, and at a rate of 0.1 s per well to ensure that consistent and accurate results were obtained.

Consistency between the fluorescence and the chemiluminescence assays was observed using a range of different strains of influenza A and B viruses to determine IC_{50} values for zanamivir. The present study found that zanamivir IC_{50}s ranged from 1.0 to 7.5 nM at a fixed concentration of NA-STAR. These values corresponded well to those obtained using the fluorescence assay (1.0 to 5.7 nM) (Table 3).

In previous studies, NA assays have proven to be the most reliable and reproducible way of monitoring the susceptibility of influenza clinical isolates to zanamivir (11). Furthermore, studies by Barnett *et al.* (11) have clearly indicated that the NA enzyme assay is currently the only *in vitro* assay that is predictive of *in vivo* susceptibility to zanamivir. In this study, the chemiluminescent NA assay has proved to be especially valuable in monitoring the susceptibility of those isolates that possessed NA activity too low to be measured in the current fluorescence assay. Therefore, based on this comparison we would recommend that future susceptibility monitoring of influenza NA to zanamivir should be carried out using the NA-STAR chemiluminogenic substrate.

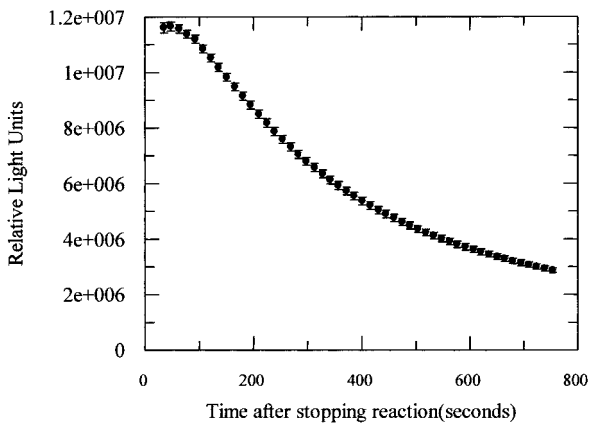

FIG. 8. Chemiluminescence decay curve. The NA-STAR assay was carried out as outlined under Materials and Methods. A concentration of NA was chosen such the S/N ratio was no more than 40/1 and diluted prior to assay in 32.5 mM Mes, pH 6.0, and 4 mM $CaCl_2$. This was achieved by initially titrating NA with NA-STAR substrate and plotting NA concentration against S/N using Grafit. Following chemiluminescent light emission, photon emissions were obtained at the times indicated on the *x* axis. The data displayed are the mean. Errors represent the mean ± SE (n = 3).

TABLE 3

Zanamivir IC_{50} Values Using the Fluorescence and the Chemiluminescence Assays

Virus (subtype + serology)	IC_{50} (nM zanamivir) (fixed [MUN] at 100 μM)	IC_{50} (nM zanamivir) (fixed [NA-STAR] at 100 μM)
B/Beijing/184/93	1.6 ± 0.3	2.9 ± 0.2
B/Harbin/7/94	5.7 ± 2.5	7.5 ± 0.9
A/Shandong/9/93 (H3N2)	2.6 ± 1.7	3.9 ± 0.7
A/Johannesburg/33/94 (H3N2)	5.5 ± 1.6	2.9 ± 0.6
A/Taiwan/1/86 (H1N1)	1.0 ± 0.4	2.9 ± 0.1
A/Texas/1/91 (H1N1)	1.3 ± 0.5	1.0 ± 0.2

Note. A selection of influenza A and B viruses was diluted 1:100 prior to assay in 4 mM $CaCl_2$ combined with either 32.5 mM Mes, pH 6.5, for the fluorescence assay or 32.5 mM Mes, pH 6.0, for the chemiluminescence assay. This dilution was decided by initially titrating the viruses with NA-STAR substrate and plotting the viral dilution against RLUs or RFUs using Grafit. The NA-STAR and MUN assays were conducted as outlined under Materials and Methods except that the concentration of MUN and NA-STAR used was 100 μM. Data were fitted and zanamivir IC_{50} values determined using Grafit. Errors represent the mean ± SE (n = 2).

REFERENCES

1. Palese, P., Tobita, K., Ueda, M., and Compans, R. W. (1974) *Virology* **61,** 397–410.
2. Hayden, F. G. (1996) *in* Antiviral Drug Resistance (Richman, D. D., Ed.), pp. 59–77, Wiley, Chichester, England.
3. Gubareva, L. V., Bethell, R., Hart, G. J., Murti, K. G., Penn, C. R., and Webster, R. G. (1996) *J. Virol.* **70,** 1818–1827.
4. McKimm-Breschkin, J. L., Blick, T. J., Sahasrabudhe, A., Tiong, T., Marshall, D., Hart, G. J., Bethell, R. C., and Penn, C. R. (1996) *Antimicrob. Agents Chemother.* **40,** 40–46.
5. McKimm-Breschkin, J. L., Sahasrabudhe, A., Blick, T. J., McDonald, M., Colman, P. M., Hart, G. J., Bethell, R. C., and Varghese, J. N. (1998) *J. Virol.* **72,** 2456–2462.
6. Blick, T. J., Tiong, T., Sahasrabudhe, A., Varghese, J. N., Colman, P. M., Hart, G. J., Bethell, R. C., and McKimm-Breschkin, J. L. (1995) *Virology* **214,** 475–484.
7. Staschke, K. A., Colacino, J. M., Baxter, A. J., Air, G. M., Bansal, A., Hornback, W. J., Munroe, J. E., and Laver, W. G. (1995) *Virology* **214,** 642–646.
8. Gubareva, L. V., Robinson, M. J., Bethell, R. C., and Webster, R. G. (1997) *J. Virol.* **71,** 3385–3390.
9. Gubareva, L. V., Matrosovich, M. N., Brenner, M. K., Bethell, R. C., and Webster, R. G. (1998) *J. Infect. Dis.* **178,** 1257–1262.
10. Hayden, F. G., Cote, K. M., and Gordon Douglas, R. (1980) *Antimocrob. Agents Chemother.* **17,** 865–870.

11. Barnett, J. M., Cadman, A., Gor, D., Dempsey, M., Walters, M., Candlin, A., Tisdale, M., Morley, P., Owens, I., Fenton, R. J., Claas, E. C. J., Rimmelzwaan, G. F., DeGroot, R., and Osterhaus, A. D. M. E. (2000) *Antimicrob. Agents Chemother.* **44,** 78–87.
12. Potier, M., Mameli, L., Belisle, M., Dallaire, L., and Melancon, S. B. (1979) *Anal. Biochem.* **94,** 287–296.
13. Bronstein, I., Voyta, J. C., Vant Erve, Y., and Kricka, L. J. (1991) *Clin. Chem.* **37,** 1526–1527.
14. Edwards, S. B., Sparks, A., Voyta, J. C., and Bronstein, I. (1994) *in* Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects (Campbell, A. K., Kricka, L. J., and Stanley, P. E., Eds.), pp. 56–59, Wiley, Chichester, England.
15. Hart, G. J., and Bethell, R. C. (1995) *Biochem. Mol. Biol. Int.* **36,** 695–703.
16. Kuhn, R., Lutz, P., and MacDonald, D. L. (1966) *Chem. Ber.* **99,** 611–617.
17. Woods, J. M., Bethell, R. C., Coates, J. A. V., Healy, N., Hiscox, S. A., Pearson, B. A., Michael Ryan, D. M., Ticehurst, J., Tilling, J., Walcott, S. M., and Penn, C. R. (1993) *Antimicrob. Agents Chemother.* **37,** 1473–1479.
18. Bronstein, I., and McGrath, P. (1989) *Nature* **338,** 599–600.
19. Jain, V. K., Magrath, I. T. (1991) *Anal. Biochem.* **199,** 119–124.
20. Van Poucke, S. O., and Nelis, H. J. (1995) *Appl. Environ. Microbiol.* **61,** 4505–4509.
21. Martin, C. S., Olesen, C. E. M., Liu, B., Voyta, J. C., Shumway, J. L., Juo, R. R., and Bronstein, I. (1997) *in* Bioluminescence and Chemiluminescence; Molecular Reporting with Photons (Hastings, J. W., Kricka, L. J., and Stanley, P. E., Eds.), pp 525–528, Wiley, Chichester, England.
22. Bronstein, I., Martin, C. S., Fortin, J. J., Olesen, C. E. M., and Voyta, J. C. (1996) *Clin. Chem.* **42,** 1524–1546.
23. Pegg, M. S., and Von Itzstein, M. (1994) *Biochem. Mol. Biol. Int.* **32,** 851–858.