Optimization of Assay Conditions fo r Dengue Virus Protease: Effect of Various Polyols and Nonionic Detergents

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Optimization of Assay Conditions for Dengue Virus Protease: Effect of Various Polyols and Nonionic Detergents

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The aim of this work was to perform a systematic study of the effect of nonionic detergents on the activity of the dengue virus NS2B-NS3 protease. To ensure a high activity of the protease, the assay procedures for the dengue virus and other flaviviral proteases published to date are performed in the presence of up to 35% glycerol, which does not represent the cellular physicochemical environment. In addition, the high viscosity of glycerol-containing solutions leads to various experimental problems in miniaturized assays. Using an internally quenched peptide substrate, the authors show that glycerol is not essential for enzymatic activity if certain nonionic detergents are added to the assay buffer. In addition, nonionic detergents may help to avoid false-positive screening results caused by "promiscuous" inhibitors. Other polyalcohols can substitute glycerol and have less effect on the viscosity of the assay buffer. The assay was used to screen a compound library and allowed the identification of small-molecular nonpeptidic inhibitors of dengue NS3 protease. Finally, the authors discuss the mode of action of nonionic detergents and the influence that they may have on the conformational properties of the NS2B-NS3 protease. (*Journal of Biomolecular Screening* 2009:1102-1108)

Key words: dengue virus, protease, high-throughput screening, promiscuous inhibitors, nonionic detergents

INTRODUCTION

THE DENGUE (DEN) VIRUS BELONGS TO THE GENUS *Flaviviridae* and is closely related to the Hepaci viruses (e.g., hepatitis C virus). Recently, dengue virus and other flaviviruses such as West Nile virus (WNV) and yellow fever virus started to spread into the Northern Hemisphere because of global warming and increasing international traveling. There are currently no antiviral therapies or vaccinations for dengue virus available.

The dengue virus is an enveloped virus containing a single-stranded, positive-sense 11-kb RNA genome. The genome encodes for a polyprotein precursor, which consists of 3 structural proteins (C, capsid; prM, membrane; E, envelope) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5; **Fig. 1**). The polyprotein precursor is processed by host proteases (signalase and furin) and also by the viral NS2B-NS3 protease.[1]

In vivo, the lipophilic NS2B-cofactor is associated with the membrane of the endoplasmic reticulum (ER), whereas the NS3 is located in the cytosplasma.[2-4] For optimum activity, the NS3 depends on association with the cofactor domain.[5,6] Because of its importance in the viral life cycle, the protease is an interesting target for development of new antiviral drugs. Structural and other biochemical features such as substrate specificity are quite similar for the various, pathogenetically relevant flaviviral proteases, which offer a perspective for broad-spectrum antiviral compounds that target the individual proteases.

Various fluorogenic and chromogenic assays have been developed especially for the DEN and WNV protease. Some substrates contain a cleavable, fluorogenic, or chromogenic moiety such as aminomethylcoumarin (AMC) or *p*-nitroaniline (*p*NA) in the P1′ position of the cleavage sequence.[7,8] In this work, we employed an internally quenched anthranilamide/nitrotyrosine substrate (Abz-NleKRRS-3-(NO_2)Y)[9,10] that conforms to the preferred cleavage site of the NS2B-NS3 protease, extending from position P4 to P1′.[10] The substrates are not only different in the assays, but also the conditions vary between distinct research groups (**Table 1**). Most frequently, a TRIS-HCl buffered system in the pH range between 8.0 and 9.5 is used. The catalytic activity in all DEN and WNV protease assays is highest at low ionic strength. Numerous research groups employ the zwitterionic detergent CHAPS at a concentration of 1 mM. In the context of inhibitor screening and testing, this ionic detergent may lead to problems by interacting with charged functionalities of the test compounds, thereby causing precipitates, leading to a decreased concentration of "free" inhibitors and false-negative results.

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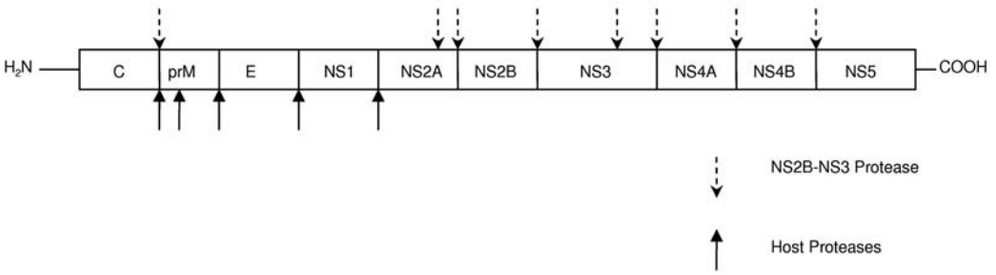

FIG. 1. Flavivirus polyprotein processing by host cell proteases (furin, signalase) and by the virus-encoded NS2B-NS3 protease.

Table 1. Overview of Assay Conditions for Flaviviral Proteases Published by Different Research Groups

Reference	*Target*	*Conc.*	*Substrate*	*Conc.*	*pH*	*Glycerol, %*	*NaCl*	*Buffer*	*Detergents*	*DMSO*
[2]	Various flaviviral proteases	0.25-0.5 mg/mL	Self-cleavage reaction	—	8.5	35	50 mM	25 mM HEPES	—	—
[8]	DV	1 µM	Ac-TTSTRR-pNA	500 µM	9	20	10 mM	50 mM Tris	1 mM CHAPS	—
[9]	DV	35-400 nM	Abz-RRRRSAGnY-NH_2	Various	9	20	—	50 mM Tris	—	—
[10]	DV (1-4)	1-3 µM	Boc-XXR-AMC/ACMC/Sbzl	150 µM	8.5	20	—	50 mM Tris	1 mM CHAPS	—
[10]	DV (1-4)	0.5-2 µM	Mca-nleKRR-XXXK(Dnp)R	100 µM	8.5	20	—	50 mM Tris	1 mM CHAPS	—
[14]	WNV	0.5-1 µM	Ac-FASGKR-pNA	500 µM	9.5	30	—	50 mM Tris	1 mM CHAPS	—
[16]	DV	Various	Abz-AKRRSQEDDnp/ Bz-ZR-AMC	Various	9	20	10 mM	50 mM Tris	—	—
[18]	WNV	500 nM	Ac-LQYTKR-pNA	250 µM	9.5	30	—	50 mM glycine/NaOH	1 mM CHAPS	—
[19]	WNV	500 nM	Ac-KPGLKR-pNA	1 mM	9.5	30	—	50 mM Tris	1 mM CHAPS	—
[20]	WNV	5 nM	Boc-GRR-AMC	—	8.5	—	50 mM	100 mM Tris	—	—
[21]	WNV	50 nM	Bz-nleKRR-AMC	20 µM	8.5	—	—	50 mM Tris	1 mM CHAPS	—
[22]	JEV + DV	1 µM	Boc-GKR-AMC	20 µM	9	—	—	100 mM Tris	—	—
[23]	YFV	100 nM	Bz-nleKRR-AMC	10 µM	8.5	20	—	50 mM Tris	1 mM CHAPS	—
[24]	DV + WNV	150 nM	Boc-GRR-AMC/ Boc-GKR-AMC	200 µM	9.5	30	13.5 mM	200 mM Tris	—	—
[25]	DV	10 nM	Pyr-RTKR-AMC/ Boc-RVRR-AMC	24 µM	8	20	—	10 mM Tris	0.005% Brij 35	—
[26]	WNV	10 nM	Boc-RVRR-AMC	24 µM	8	20	—	10 mM Tris	0.005% Brij 35	—
[27]	DV + WNV	10 nM	Pyr-RTKR-AMC	24 µM	8	20	—	10 mM Tris	0.005% Brij 35	—
[28]	WNV	10 ng/well	Pyr-RTKR-AMC	60 µM	8	20	—	10 mM Tris	0.005% Brij 35	1%
[29]	WNV	25 nM	Ac-LKKR-pNA	250 µM	9.5	30	—	50 mM glycine/NaOH	1 mM CHAPS	—
[30]	DV	50 nM	Bz-nleKRR-AMC	20 µM	8.5	—	—	50 mM Tris	1 mM CHAPS	—
This work	DV	100 nM	Abz-nleKRRS-3(NO_2)-Y	50 µM	9.0	10 (ethylene glycol)	—	50 mM Tris	0.0016% Brij 58	—

DV, dengue virus; JEV, Japanese encephalitis virus; WNV, West Nile virus; YVW, yellow fever virus. X, variable natural amino acid. Z, variable nonnatural amino acid.

Furthermore, glycerol in concentrations up to 35% is an essential component of published DEN and WNV protease assays. In an aqueous medium, glycerol modulates intra- and intermolecular hydrogen bonds and lipophilic interactions between protein and solvent.[11] Glycerol, however, increases the viscosity and can therefore cause inhomogenic mixing of the assay solution, especially when the assays are carried out in small volumes that are typically encountered in high-throughput screening (HTS) settings. In addition, a high concentration of glycerol may, by its cosmotropic activity, support the formation of multimolecular aggregates of screening compounds, thereby increasing the occurrence of false-positive results. We therefore decided to search for alternative additives that ensure a high activity of the protease while causing fewer problems with respect to liquid handling and interference with test compounds. Among other additives and experimental factors, we focused on

the nonionic detergents Brij 58, Lubrol, Tergitol NP 40, Triton X-100, Tween-60, and Tween-80, as well as on other polyols.

MATERIALS AND METHODS

Tergitol NP 40, Tween-60, Tween-80, Triton X-100, Lubrol, and Brij 58 were obtained from various commercial suppliers and from the inventory of the biopharmaceutical department at the University of Heidelberg. Ectoine, ammonium sulfate, sorbitol, and PEG 100 were also obtained from various commercial suppliers. All amino acids and the resins were obtained from Sigma-Aldrich, Germany and Bachem GmbH, Germany. Inhibitors were taken from our in-house collection. All other chemicals and biochemicals used were from Merck and were of analytical grade.

The internally quenched DEN NS2B-NS3 protease substrate Abz-NleKRRS-3-(NO_2)Y was synthesized by solid-phase synthesis[12] on Rink amide resin according to the Fmoc-protocol.[13] It was purified by preparative high-performance liquid chromatography (HPLC) using an ÄKTA Purifier, GE Germany, with a RP-18 chromatography column (Lobar®, Merck, Germany). The mobile phase consisted of MeOH and H_2O/0.1% trifluoroacetic acid (TFA) following a gradient of 20% to 100% MeOH in water with a flow rate of 1.5 mL/min. The purity of the substrate was assessed by HPLC and found to be higher than 95%. The identity was confirmed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The screening library consisted of the Klein group in-house collection and the inventory of Endotherm GmbH.

The dengue protease gene was synthesized by a commercial supplier. The expression construct comprised the hydrophilic core sequence of the NS2B cofactor linked via a flexible glycine linker to the NS3 protease domain. The linker consists of 8 glycine residues and 1 serine residue (GGGGSGGGG).[8,14] The dengue protease gene was cloned into the *Nco*I-*Sac*I site of the pET-28a vector (Novagen, San Diego, CA) and expressed in *Escherichia coli* BL 21 λ DE 3 cells. The protein was then purified by Ni^{2+}-affinity column chromatography to >95% purity. Stocks of purified protein were stored at –70 °C in 100 mM Tris-HCl (pH 7.9), 50 mM NaCl, and 50% glycerol.

Continuous assays were performed on a BMG Labtech Fluostar microtiter fluorescence plate reader using black 96-well V-bottom plates from Greiner (Monroe, NC). The excitation wavelength was 320 nm, and the emission wavelength was 420 nm. The assay was carried out in a final volume of 100 µL and at following conditions: 100 nM enzyme, 100 µM substrate, and 50 mM Tris (pH 9.0). Different assay conditions were chosen with 5% to 30% glycerol, ethylene glycol, or propylene glycol, respectively, or one of the following nonionic detergents: Brij 58, Lubrol, Tergitol NP 40, Tween-60, Tween-80, and Triton X-100. The concentrations of the detergents were varied and were always below their respective critical micelle concentration (CMC; **Table 2**). The reaction was initiated by addition of the substrate, and proper mixing was ensured by aspiration into the micropipette tip and repeated (5 times) circular mechanical stirring with the tip. The increasing fluorescence signal was monitored for 15 min with a 50-s time interval between individual measurements. The activity of the enzyme was determined as the initial slope of the fluorescence signal (relative fluorescence units/second, RFU/s). In the inhibitor screenings, the substrate concentration was 50 µM. The inhibitors were preincubated with the protease for 15 min prior to initiation of the reaction by addition of the substrate. The K_m was determined using GraFit Version 5 (Erithacus Software, Surrey, UK). The K_m value for the internally quenched peptide substrate is 55.2 µM. Details on the kinetic characterization of the substrate and the most potent inhibitor found in the screening are given in the supporting information. The K_i of the inhibitor was calculated according to the Cheng-Prusoff equation. The Z′ factors of the nonoptimized and the optimized assay were calculated as described by Zhang et al.,[15] based on a sample size of 50 positive and 50 negative controls. The Z factor for the compound screening was calculated accordingly, based on the screening results of the Endotherm library (sample size 232) and 10 controls.

Table 2. Critical Micelle Concentrations (CMC) of Different Ionic and Nonionic Detergents

Detergent	*CMC [mM]*	*Ref.*
Brij 58	0.08	31
Lubrol	0.1	32
Tergitol NP 40	0.23	33
Triton X-100	0.024	31
Tween-60	0.021	31
Tween-80	0.010	31
CHAPSO	8	31

RESULTS

Using polyols as additives, we observed the highest enzymatic activity at a high concentration. As shown in **Figure 2**, the substitution of glycerol by other polyols, like ethylene glycol or propylene glycol, increases the activity of the protease to nearly the same extent. The advantage of this substitution is the lower viscosity of the assay components, which can be handled more reliably, and of the resulting final assay mixture. We also studied the effect of nonionic detergents on the activity of the DEN protease. All nonionic detergents were assayed at concentrations well below their CMC. The most prominent results were achieved with Brij 58, Lubrol, and Tergitol, whereas Tween-60, Tween-80, and Triton X-100 were less effective (**Fig. 2**).

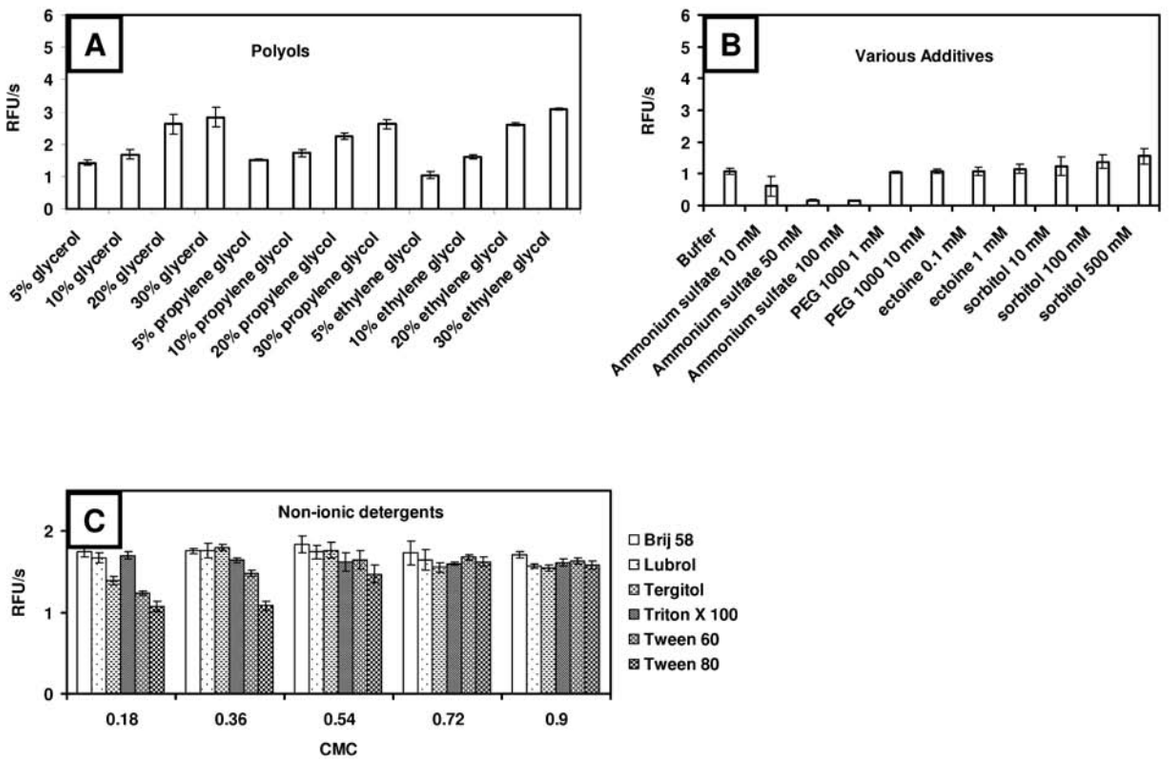

FIG. 2. Effect of different concentrations of polyols, nonionic detergents, and other additives on the activity of NS2B-NS3 protease. (**A**) Polyols. (**B**) Various additives. (**C**) Detergents. All assays were performed in triplicate, $n = 3$. Error bars represent the standard deviation from the mean.

The influence of the detergents on NS3 is concentration dependent. For Brij 58, Lubrol, Triton X-100, and Tergitol, enzyme activity decreases somewhat as the CMC is approached. Tween-60 and Tween-80 show the opposite effect, with the highest activity close to the CMC.

Brij 58, Lubrol, and Tergitol NP 40 give optimum activities at concentrations between 0.18-0.54 CMC and 0.3-0.4 CMC, respectively.

The effect was also observed at lower pH values (data not shown). Generally, the protease shows lower activity at acidic pH,[16] but the effect of the nonionic detergents is similar to those at pH 9.0.

We also studied the effect of other additives that are known to influence the tertiary structure of proteins in solution. As shown in **Figure 2**, none of these additives achieves the effect of the polyols or the nonionic detergents on the activity of the NS3 protease.

The combination of polyols with the 2 best nonionic detergents results in a further increase of NS3 protease activity. Also, the ionic detergent CHAPSO shows, in combination with the polyols, a higher activity of the protease in comparison to the detergent-free assay (**Fig. 3**). CHAPSO was used in a concentration of 1 mM similar to the published DEN protease assays with CHAPS (**Table 1**).[10]

For all further investigations, we chose a combination of 10% ethylene glycol and 0.0016% Brij 58 because this composition ensures a high activity of the NS2B-NS3 protease as well as reliable and easy handling in comparison to the standard procedure with 20% glycerol.

As a characteristic assay parameter, the Z′ factor provides information as to how accurately positive and negative signals can be separated. Here the negative control signal corresponds to the fluorescence signal of the uncleaved substrate, whereas the positive signal results from the substrate cleavage by uninhibited enzyme. In the glycerol-containing assay, the Z′ factor was 0.66, whereas the new conditions described here result in an improved Z′ factor of 0.84. This indicates that the assay conditions are well suited for screening purposes. We also studied the potential of these assay conditions to prevent inhibition of NS3 by nonselective or "promiscuous" inhibitors in comparison to glycerol. Curcumin and nordihydroguaiaretic acid were employed as exemplary promiscuous inhibitors[17] because these compounds inhibit NS3 but also a variety of other enzymes, which are routinely assayed in our group in the lower

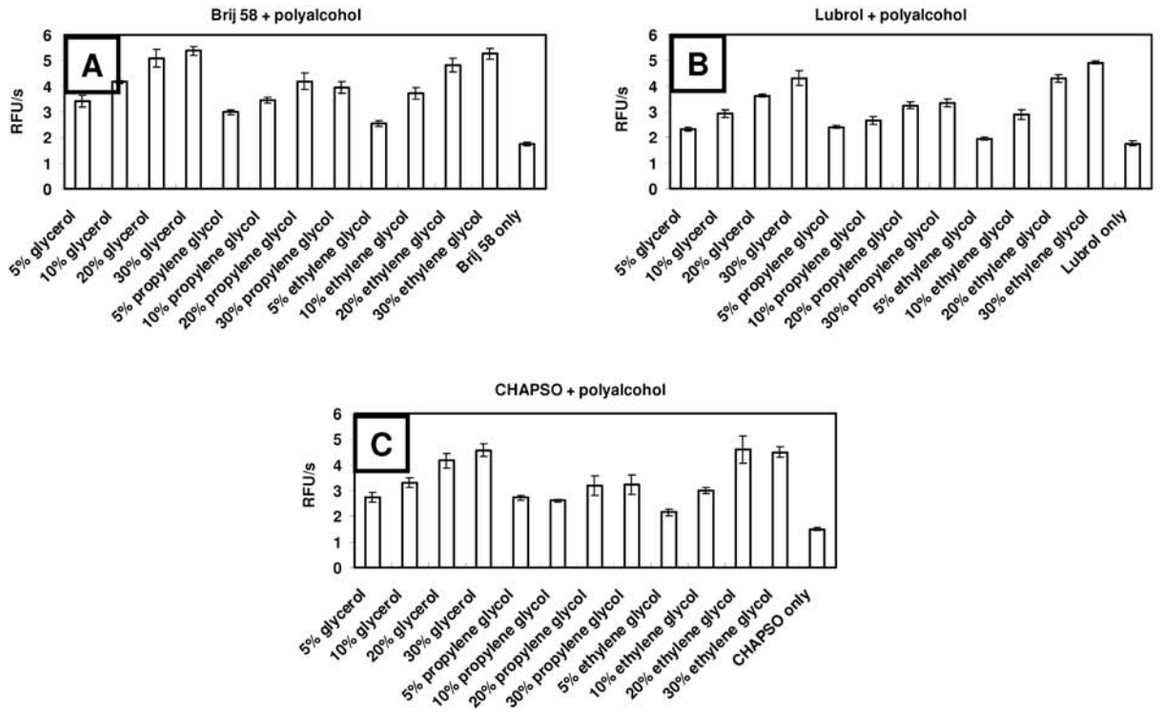

FIG. 3. Combined effect of polyols and the 3 most effective detergents: (**A**) Brij58 and polyol, (**B**) Lubrol and polyol, and (**C**) CHAPSO and polyol. All assays were performed in triplicate, $n = 3$. Brij 58, Lubrol, and CHAPSO were used in concentrations of 14 μM, 36 μM, and 1 mM, respectively. Error bars represent the standard deviation from the mean.

micromolar concentration range. As shown in **Figures 4A, B**, the combination of 10% ethylene glycol and Brij 58 decreases the inhibitory effect of these promiscuous inhibitors. In the assay with glycerol, 100 μM nordihydroguaiaretic acid causes an inhibition of nearly 70%, whereas the modified assay conditions decrease the inhibition to about 30%. The same effect occurred in the assay using curcumin. In the assay with glycerol, curcumin induces nearly 100% inhibition. In contrast, 10% ethylene glycol and Brij 58 decrease the inhibitory effect to 70%.

To find new potent inhibitors for the dengue NS3 protease, we screened 2 compound libraries for activity against the protease. The libraries were the collection of Endotherm GmbH containing about 250 small organic molecules and the Klein group in-house substance library consisting of about 200 commercially available drugs, natural products, and other proprietary compounds.

All compounds were dissolved in DMSO and preadded as duplicates into 96-well plates. Subsequently, the other components of the assay were added to yield a concentration of test compounds of 50 μM. Every measurement was performed twice to minimize fluctuations. The initial velocities were related to uninhibited control experiments. Potent hits were studied in detail (concentration-effect curves, time dependency, stability of the test compound, additional HPLC-based assay etc.). This led to the identification of, to our knowledge, the most potent small-molecule inhibitor (Endo 5) of the dengue NS3 protease reported to date, with an inhibitory constant of $K_i = 13.1$ μM. The kinetic characterization of Endo 5 indicates an uncompetitive binding mode (cf. the supporting information for detailed data). Further characterization of this compound is currently performed in a cell culture model of dengue virus replication. The Z factor for the compound screening was 0.55.

DISCUSSION

We investigated the influence of polyols and nonionic detergents on an NS2B-NS3 fluorescence assay. Glycerol, a main component in various NS2B-NS3 assays published in the literature, can be favorably replaced by other polyols. The 3D structure of the solvated NSB-NS3 protease—which is in a different physicochemical surrounding compared to the membrane-bound species that is relevant under in vivo conditions—is obviously stabilized in a "productive" conformation by the high glycerol content of the commonly used assay buffers. The use of certain detergents, which probably (partially) resemble the membrane environment encountered by NS3 in vivo, where the protease is localized in close proximity to the membrane of the ER, results in even higher enzymatic activities. We therefore suggest that

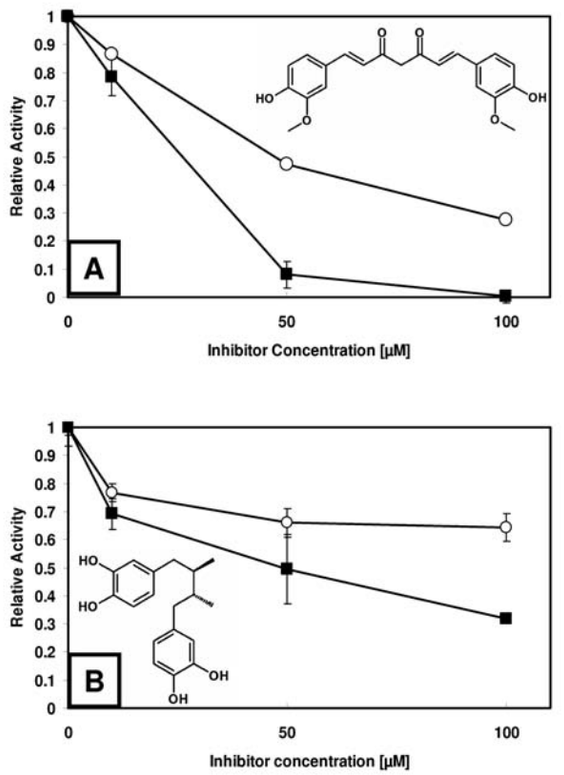

FIG. 4. Inhibition of NS2B-NS3 by the promiscuous inhibitors (**A**) curcumin and (**B**) nordihydroguaiaretic acid in 14 µM Brij 58/10% ethylene glycol (○) and 20% glycerol (■).

the presence of nonionic detergents provides a more "native" state of DEN NS3 and possibly other flaviviral proteases, which obviously has implications for in vitro/in vivo correlations.

In addition, the replacement of the highly viscous glycerol by the less viscous ethylene glycol in a lower concentration has the added values of easy liquid handling and more reliable mixing. One may also speculate that polyols favor the formation of multimolecular aggregates of "promiscuous" inhibitors, thereby leading to false-positive results. The combination of ethylene glycol with Brij 58 as nonionic detergent reduces the inhibitory potency of such promiscuous inhibitors, thereby decreasing the false-positive rate.

The optimization of the assay conditions improves the Z′ factor of the assay from 0.66 to 0.84. Further evidence for the good performance of the assay protocol comes from the screening of 2 compound libraries (Z factor 0.55), which resulted in the identification of a lead structure that will be used for further inhibitor development.

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