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Antipneumococcal activity of neuraminidase inhibiting artocarpin

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ABSTRACT

Streptococcus (*S.*) *pneumoniae* is a major cause of secondary bacterial pneumonia during influenza epidemics. Neuraminidase (NA) is a virulence factor of both pneumococci and influenza viruses. Bacterial neuraminidases (NAs) are structurally related to viral NA and susceptible to oseltamivir, an inhibitor designed to target viral NA. This prompted us to evaluate the antipneumococcal potential of two NA inhibiting natural compounds, the diarylheptanoid katsumadain A and the isoprenylated flavone artocarpin. Chemiluminescence, fluorescence-, and hemagglutination-based enzyme assays were applied to determine the inhibitory efficiency (IC_{50} value) of the tested compounds towards pneumococcal NAs. The mechanism of inhibition was studied via enzyme kinetics with recombinant NanA NA. Unlike oseltamivir, which competes with the natural substrate of NA, artocarpin exhibits a mixed-type inhibition with a K_i value of 9.70 μM. Remarkably, artocarpin was the only NA inhibitor (NAI) for which an inhibitory effect on pneumococcal growth (MIC: 0.99–5.75 μM) and biofilm formation (MBIC: 1.15–2.97 μM) was observable. In addition, we discovered that the bactericidal effect of artocarpin can reduce the viability of pneumococci by a factor of >1000, without obvious harm to lung epithelial cells. This renders artocarpin a promising natural product for further investigations.

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Introduction

S. pneumoniae is responsible for the majority of pneumonia cases and the death of about 1.2 million young children worldwide each year (18% of all deaths of children under the age of five) (Black et al., 2010; Krzysciak et al., 2013). Spreading of *S. pneumoniae* in the nasopharynx and surrounding tissues causes the clinical manifestation. The diseases range from mild upper respiratory tract infections, such as acute otitis media, sinusitis, and pneumonia, to severe and potentially life-threatening conditions, such as meningitis and sepsis, by bacterial invasion of the bloodstream (Simell et al., 2012). Additionally, a lethal synergism between pulmonary coinfections with influenza virus and *S. pneumoniae* has been established, accounting for the excess mortality during influenza epidemics and pandemics whereat pneumococcal NAs were found to support viral release and spread in the lung (Kash et al., 2011; McCullers and Bartmess, 2003).

Pneumococcal NAs (NanA, B, and C) belong to a wide range of surface-associated proteins interacting with eukaryotic cells, extracellular matrix proteins, and serum proteins (Lofling et al., 2011). They catalyze the removal of terminal sialic acid residue from various glycoconjugates on cell surface (Taylor, 1996), by which means they reveal receptors for bacterial adhesion (King et al., 2006). They also promote upper (Tong et al., 2000) and lower (Orihuela et al., 2004) airway colonization, biofilm formation, and mucosal infection (Brittan et al., 2012; King et al., 2006; Soong et al., 2006). The released sialic acids serve as a carbon source for the bacteria and represent a trigger for biofilm formation (Trappetti et al., 2009). In

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Table 1
The *S. pneumoniae* strains studied with both genetic and phenotypic identification of NanA activity.

Strain	Source	*nanA*	*nanB*	*nanC*	NA activity[a]
DSM20566	Reference strain	×	×	–	×[b]
Recombinant	NanA of DSM20566	×	–	–	×[b]
DSM14378	Reference strain	×	×	–	×
D39	Reference strain	×	×	–	×[b]
CF6937	Cystic fibrosis	×	–	×	×
CF8919	Cystic fibrosis	×	×	–	×
BC7326	Blood culture, sepsis	×	×	×	×
BC57	Blood culture, sepsis	×	×	–	×
PN8828	Pneumonia	×	×	–	×
CJ9400	Conjunctivitis	×	×	–	×[b]

[a] NA activity was proved in the FL- and CL-based assays.
[b] Lectin-based HA assay was additionally performed to demonstrate the NA activity.

addition, pneumococcal NAs contribute critically to inflammation and mortality associated with sepsis (Chen et al., 2011).

The essential roles of NAs during coinfection with influenza viruses and in pathogenesis of pneumococcal strains render them an attractive target for therapeutic intervention (Taylor, 1996). Blocking NA activity with small-molecule inhibitors in the intestinal perforation model of sepsis led to a substantial reduction of the inflammatory response and subsequent morbidity (Chen et al., 2011; Paulson and Kawasaki, 2011). Administration of the influenza virus-specific neuraminidase inhibitor (NAI) oseltamivir interrupted the lethal synergism between influenza virus and *S. pneumoniae* and prevented excess mortality from secondary bacterial pneumonia in a mouse model (McCullers and Bartmess, 2003). Currently, there are only two influenza NAIs (zanamivir and oseltamivir) prescribed worldwide for the treatment and control of influenza (Grienke et al., 2012). Their inhibitory potencies are either weak (zanamivir) or medium (oseltamivir) (Gut et al., 2011) to pneumococcal NA.

Recently, we discovered the diarylheptanoid katsumadain A and the isoprenylated flavone artocarpin as novel NAIs acting against influenza viruses (Grienke et al., 2010; Kirchmair et al., 2011). In the present study, we evaluated the antipneumococcal potential of both natural product NAIs. We analyzed their inhibitory effect on pneumococcal NA and performed enzyme kinetic studies to understand the molecular mechanism of their inhibition of NanA. In addition, we investigated whether these NAIs affect the bacterial growth, adsorption, biofilm formation, and viability.

Material and methods

Compounds

Oseltamivir carboxylate GS4071 (oseltamivir; Roche AG, Basel, Switzerland), zanamivir (GlaxoSmithKline, Brentford, UK), DANA (2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid), and rifampicin (both purchased from Sigma-Aldrich, Deisenhofen, Germany) were dissolved in water as 10 mM stock solutions. Rifampicin was stored at −20 °C. Artocarpin (Quality Phytochemicals LLC, East Brunswick, NJ, USA) and katsumadain A, previously isolated from the seeds of *Alpinia katsumadai* Hayata (Grienke et al., 2010), were dissolved in DMSO as 10 mM stock solutions and stored at 4 °C. Their HPLC purity revealed to be >98%.

Bacterial strains, cells, media, and pre-culture conditions

Six *S. pneumoniae* clinical isolates were collected from patients with different symptoms (Table 1). Two reference strains DSM20566 (serotype 1, ATCC 33400) and DSM14378 (serotype 5, ATCC 6305) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Heidelberg, Germany). *S. pneumoniae* D39 (serotype 2) was kindly provided by ZIK Septomics (Jena, Germany).

All nine strains used in this study were grown on Columbia blood agar plates supplemented with 5% sheep blood (Becton Dickinson GmbH, Heidelberg, Germany) at 37 °C in an atmosphere enriched with 5% CO_2 overnight. During pre-cultivation, bacteria were grown in brain heart infusion broth (BHI) with slightly shaking at 37 °C for 4 to 5 h. This incubation time corresponded with the mid-exponential phase of bacterial growth as evaluated exemplarily with two reference strains (data not shown).

To study growth and biofilm inhibition by the test compounds, samples of precultured pneumococci were diluted in BHI to a McFarland of 0.5 (1.5×10^8 cfu/mL). BHI medium was used for all assays, except for determination of biofilm due to the broad usage of tryptic soy broth (TSB) in pneumococcal biofilm research (Oggioni et al., 2006; Trappetti et al., 2009; Yadav et al., 2012).

Human lung carcinoma cells (A549; Institute of Molecular Virology, University of Münster, Germany) were maintained in Dulbecco's Modified Eagle Medium (Lonza Group Ltd, Basel, Switzerland) supplemented with 10% fetal calve serum (PAA Laboratories GmbH, Cölbe, Germany). Cells were cultivated at 37 °C with 5% CO_2.

Genomic DNA isolation, nanA gene amplification, and sequencing

Genomic DNA of nine *S. pneumoniae* strains was isolated from bacterial cells using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The ∼1.5 kb 16S rRNA gene was amplified and sequenced using conventional primers 27F and 1492R devised by Weisburg et al. (1991) to confirm their identity as *S. pneumoniae* (data not shown). NA gene fragments were amplified with PCR Taq Core Kit 10 (MP Biomedicals, Eschwege, Germany). For *nanA* amplification primers were designed based on a pneumococcal *nanA* gene in GenBank (X72967) (Table S1), and for *nanB* and *nanC* we used primers described previously (Burnaugh et al., 2008). Amplification conditions were: 5 min initial denaturation, followed by 38 cycles of 30 s at 96 °C, 30 s at 50–60 °C, and 60 s at 72 °C. Products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by Eurofins Food GmbH (Ebersberg, Germany). The *nanA* of DSM20566 was completely sequenced (GenBank accession number KJ850445).

Preparation of precipitated pneumococcal total proteins for assays

Out of 10 mL of liquid pneumococcal culture in BHI medium, 200 μL were taken to extract the total proteins from cells. Upon addition of 1.8 mL absolute ethanol, the sample was kept overnight at −20 °C. After centrifugation for 10 min, precipitated total protein was washed with ice-cold 70% ethanol and redissolved either in 50 μL PBS buffer for cell-based assay or in 50 μL buffer (32.5 mM MES, 4 mM calcium chloride, pH 6.5) for enzyme-based assays.

Expression and purification of rNanA of S. pneumoniae *DSM20566 from* E. coli

In order to obtain the *nanA* from *S. pneumoniae* strain DSM20566, primer pair NA_116aaNdeI_fw (5′-TGCACGACATATGGAAAATGTC-3′) and NA_Cter_XhoI_rv (5′-TCAAATCTCGAGAATTCTTCTCT-3′) were applied to amplify this gene. The NdeI/XhoI double-digested PCR product was then ligated to the *E. coli* expression vector pET-28a. The constructed plasmid encodes an N-terminal 6× His-tagged 886aa protein. A 150 mL *E. coli* culture of BL21(DE3)/pTNA20566-116aa was used to synthesize the enzyme. Gene expression was induced by IPTG

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