FINAL REPORT

The Serine Protease, Po;ynucleotide-stimulated – NTPase and the RNA Helicase multiple enzymatic function of nonstructural protein NS3 – Model Biochemical Tragets essential for Replication and Muturation of Dengue and Japanese Encephalitis Virus

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ผู้วิจัย

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สังกัด

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

BRG 4980008

FINAL PROJECT REPORT

THE SERINE PROTEASE, POLYNUCLEOTIDE-STIMULATED NTPase AND THE RNA HELICASE MULTIPLE ENZYMATIC FUNCTIONS OF NONSTRUCTURAL PROTEIN NS3 – MODEL BIOCHEMICAL TARGETS ESSENTIAL FOR REPLICATION AND MATURATION OF DENGUE AND JAPANESE ENCEPHALITIS VIRUS

PRINCIPAL INVESTIGATOR: GERD KATZENMEIER

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Gerd Katzenmeier

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ABSTRACT

Project Code: BRG 4980008

Project Title:

THE SERINE PROTEASE, POLYNUCLEOTIDE-STIMULATED NTPase AND THE RNA HELICASE MULTIPLE ENZYMATIC FUNCTIONS OF NONSTRUCTURAL PROTEIN NS3 – MODEL BIOCHEMICAL TARGETS ESSENTIAL FOR REPLICATION AND MATURATION OF DENGUE AND JAPANESE ENCEPHALITIS VIRUS

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Summary:

The project detailed herein describes the ongoing work on the biochemical characterization of the NS3 proteases of human-pathogenic flaviviruses such as dengue and Japanese encephalitis virus. The project **a**) analyzes structure-activity relationships within the active site of the NS2B-NS3 protease by structure-guided mutagenesis, **b**) explores the predictive potential of proteochemometric models for the analysis of enzyme-ligand interaction by using the NS3 serine proteases as demonstration case for virtual inhibitor design, **c**) establishes methods for the purification and assay of the NS3 NTPase and RNA helicase activities and **d**) describes the purification and preliminary activity assay for the NS3 protease from Japanese encephalitis virus.

Keywords: dengue virus, NS2B-NS3, protease, NTPase, helicase, assay, ligand, proteochemometrics

SUMMARY

1. Within this project we have been able to initiate work on the extensive characterization of the flaviviral NS2B-NS3 two-component proteases, mainly from dengue virus but also from Japanese encephalitis virus, and to establish a research infrastructure, expertise and technical facilities for the molecular biological and biochemical investigations. Despite a comparatively low number of MSc students and in the absence of additional technical personnel, we have been able to produce a number of international publications.

2. We were able to demonstrate that despite obvious similarities in overall structure, the NS3 proteases from 4 dengue virus serotypes posses a number of discrete properties such as expression yield, pattern of product formation, refolding kinetics and enzymatic reactivity. These data could suggest that subtle structural variations modulate the kinetics of virus replication *in vivo* and also would affect the susceptibility to future protease inhibitors (*Biochimica et Biophysica Acta, 2008, **1780**, pp. 989-994*).

3. We have established methods for the cloning, purification and assay of the dengue virus NS3 NTPase and RNA helicase. The NS3h domain was purified by metal chelate affinity chromatography followed by renaturation of precursors, mediated by artificial chaperone-assisted refolding which yielded the active helicase.

4. The NS2B-NS3pro protease of Japanese encephalitis virus was cloned in *E. coli*, overexpressed and purified. The sequence of NS2B/NS3 from JEV was obtained by synthesis of the full-length gene and the active protease complex NS2B(H)/NS3 pro was generated by SOE-PCR. This analysis yielded – for the first time – kinetic parameters for K_m and k_{cat} of the JEV NS3 protease. The data also revealed differences between the dengue virus NS3 protease and JEV NS3, although both enzymes had comparable requirements for pH and ionic strength.

5. In collaboration with Prof. Jarl E. S. Wikberg, Dept. of Pharmaceutical Biosciences, Uppsala University, Sweden, we have completed a significant study on the development and refinement of proteochemometric models for the dengue virus NS3 protease (*Bioorg. Med. Chemistry 2008, **16**, pp. 9369-9377*).

6. Important determinants of substrate binding and catalysis of the dengue virus NS3 protease were identified in a structure-guided mutagenesis study. We were able to confirm that our kinetic data were essentially in agreement with a functional role of the selected residues for substrate binding and/or catalysis (*Journal of Biomedical Science, 2010, **17**, pp. 68-75*).

INTRODUCTION

The mosquito-borne 'Flavivirus' genus includes 72 known members of human pathogens of global distribution and prevalence representing a massive burden for public health care. At present, there is no known chemotherapy for any flavivirus and control of these diseases through vaccination is currently elusive. Dengue virus is the etiologic agent of dengue fever and the global pandemic has dramatically intensified over the past decade with approximately 100 million people infected in more than 100 tropical and subtropical countries [1,2]. Japanese encephalitis virus (JEV), a member of the *Flaviviridae* family, is a mosquito-borne neurotropic flavivirus and causes severe central nerve system diseases. The flavivirus-borne diseases drain significant financial resources in developing countries. However, significant progress has been made in the identification of viral enzymes as potential targets for the design, synthesis and evaluation of antiviral drugs which are eventually useful for a causative and/or preventive treatment of flavivirus diseases.

Dengue virus, a member of the *Flaviviridae* family, is a small, spherical, enveloped, positive single strand RNA virus that is transmitted to humans by mosquitoes of the species *Stegomyia aegypti* (formerly *Aedes*). All 4 serotypes of the virus (DEN-1, 2, 3 and 4) can cause a spectrum of clinical symptoms including mild dengue fever (DF) and more severe forms of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). An increase of geographical spread, incidence and severity of diseases over the past decade has now stimulated intensive efforts to develop effective antiviral therapeutics which are eventually useful for the prevention and cure of dengue virus infections. The development of small molecule drugs directed at inhibition of replication and maturation of the virus is now considered as promising route for the treatment of acute dengue diseases [*for review see [3-5] and references herein*].

The dengue virus NS3 protease, a member of the flavivirin enzyme family (EC 3.4.21.91), is located in the N-terminal 184 residues of the multifunctional 69 kDa NS3 protein and contains a functional catalytic triad consisting of H51, D75 and S135 (in DEN-2) [6]. In addition to the serine protease, the NS3 protein contains enzymatic activities of a nucleoside triphosphatase, a 5′ - RNA triphosphatase (RTPase) and a RNA – stimulated RNA helicase [7,8]. The NS3 protease catalyses the post-translational cleavage of the viral polyprotein precursor in the non-structural region at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 sites and at additional sites within the viral capsid protein, NS2A, NS4A and within a C-terminal region of NS3 itself [9-13]. The overall conformation of the dengue virus NS3 protease displays the b-barrel conformation typical for serine proteases, although the viral enzyme appears to possess higher compactness with short or absent loop structures and a relatively shallow substrate binding site [14]. The presence of a small hydrophilic core segment of approximately 40 residues, commonly designated NS2B(H), within the small 14 kDa NS2B cofactor is required for optimal activity of the NS3 protease [15-17]. Proteolytic autoprocessing at the NS2B/NS3 site generates a non-covalent adduct between NS2B(H) and NS3 which is catalytically active with substrates supplied in trans cleavage reactions [18]. Detailed substrate specificity studies have established that the cleavage junctions in the viral polyprotein consist of pairs of dibasic amino acids such as RR, RK and KR at the P1 and P2 positions. Small, non-branched amino acids such as S are preferred at the P1′ position of the dengue virus cleavage site, whereas the preferred P1′ residue of the WNV NS3 protease is G [19-21]. Theoretical molecular interactions between the active site of the NS3 protease and the peptide substrate were largely consistent with data obtained from substrate profiling studies [22]. Crystallographic studies of flaviviral proteases including the West Nile Virus (WNV) and dengue virus in complex with a partial NS2B cofactor and substrate-mimetic inhibitors such as aprotonin have provided evidence for major structural

reorganizations of the active site pockets caused by insertion of a β-barrel of the NS2B cofactor and an "induced fit" mechanism of catalysis in the presence of authentic protein substrates [23]. Based on a homology-modelled structure of the WNV NS3 protease, residues within the S1 and S2 pockets critical for enzyme-substrate interaction were identified by analysis of catalytic activity of mutant proteases with a synthetic peptide substrate [24]. Structural data obtained recently for a WNV NS2B-NS3pro protease in complex with a substrate-based tripeptide inhibitor have revealed a catalytically competent oxyanion binding site formed by two residues, G133 and S135, and substitution of the active-site nucleophile serine by alanine does not result in a disruption of the oxyanion conformation [25]. It is noteworthy that also in the presence of ligands without a P1′ residue the active conformation of the oxyanion hole is adopted by the viral protease.

A high conservation of sequences within the faviviral proteases suggests that specificity characteristics found for the WNV protease could also be of relevance for the closely related dengue virus NS3 protease. Despite their overall similarities, the NS3 proteases from dengue virus, Japanese encephalitis virus and WNV exhibit different substrate specificities, suggesting a distinct organization of their respective active site conformations [21]. An earlier study has described extensive mutagenesis within the dengue virus NS3 protease for ultraconserved residues among flaviviral proteases and these residues were putatively involved in catalysis or substrate binding [26]. However, activity of the mutant proteases was assayed by SDS-PAGE analysis of autoproteolytic cleavage of the NS2B-NS3 precursor *in vivo*. Although this approach yielded semiquantitative data for activity of the mutant enzymes, it did not provide precise numerical values for the kinetic activity of the mutant proteases with substrates supplied for *trans* cleavage reactions. Moreover, a number of residues such as L115, S163 and I165 have not been

included in that investigation as their possible role for enzyme activity was suggested later by data from structural experiments [14, 23].

The project proposed in TRF grant application BRG 4980008 aimed at generating structural and mechanistic data for the NS2B/NS3 serine proteases from human-pathogenic flaviviruses with a view to the rational design of inhibitors which are eventually useful as lead candidates for drug discovery. We have also proposed to extend the investigations to the enzymatic functions of the nucleoside triphosphatase (NTPase) and the RNA helicase of the multifunctional NS3 protein. In the previous report we have described the methods and strategies required to achieve the proposed objectives.

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SUMMARY OF RESULTS AND DISCUSSION

The grant application for this project continued previously approved project BRG4680006 (ENZYME-SUBSTRATE INTERACTIONS, COFACTOR REQUIREMENT AND INHIBITOR DEVELOPMENT WITH THE DENGUE VIRUS SERINE PROTEASE COMPLEX NS2B/NS3, A MODEL TARGET ENZYME ESSENTIAL FOR FLAVIVIRUS REPLICATION AND PATHOGENESIS). Our work is directed towards establishing research infrastructures, training, education of students and scientific data collection by using the NS3 serine proteases of pathogenic flaviviruses as demonstration case for novel therapeutic targets of neglected and/or emerging pathogens with relevance to human health. The project also aims at establishing international collaborations with research laboratories having extensive experience in the field and thereby contributes to means for the prevention and possible therapeutic options for the increasing burden of flaviviral infections.

The main objectives for this phase of investigations were:

i) cloning, overexpression in recombinant *E. coli*, purification and enzymatic analysis of the NS2B-NS3 serine protease complex from Japanese encephalitis virus (JEV). The NS3 protease from JEV appears to be structurally very similar to the closely related dengue virus NS3 protease, however, we expected subtle differences in substrate specificity and inhibition profiles relevant to the design of potential inhibitors. Moreover, infection with JEV represents a

severe clinical situation with a relatively high fatality rate (approx. 30%) where the use of antiviral drugs would be urgently required;

ii) demonstration of a functional interaction between the serine protease domain and the NTase/RNA helicase domains of the dengue virus NS3 protein involved in the regulation of its respective activities;

iii) evaluation of proteochemometric models for enzyme-substrate interaction of the dengue virus NS3 protease by assay of the enzymes from 4 serotypes with libraries of synthetic peptide inhibitors;

iv) continuing work on biochemical properties of the NS3 proteases from 4 dengue virus serotypes. A comparative biochemical analysis of the NS3 proteases from 4 dengue virus serotypes has been initiated within in the previously funded project, however, the data which were collected at that time appeared to be too preliminary for publication; and

v) identification of key residues involved in substrate recognition and catalysis of the dengue virus NS3 protease by site directed mutagenesis and enzymatic assays.

We describe in this report that within the period of funding we could achieve publishable results for some of above mentioned objectives, while other subprojects (especially the generation of PCM models and inhibitor screening as well as enzymatic analysis of the JEV NS3 protease and the dengue virus NS3 NTPase/helicase) are not completed at present as these subprojects are extremely time- and labour-intensive.

I) CLONING, PURIFICATION AND ASSAY OF THE NS2B-NS3 PROTEASE FROM JAPANESE ENCEPHALITIS VIRUS

Currently, there is no crystallographic structure of the JEV NS2B-NS3 protease complex available. Although the overall 3-dimensional structures (as obtained by modeling) and cleavage site preferences of JEV NS3 protease appear to share similar characteristics with the dengue virus NS3 protease, it is conceivable that subtle mechanistic and/or regulatory variations exist, which are involved in a modulation of enzymatic activity and polyprotein processing. In addition, the JEV protease is an attractive model to test the currently proposed concept of “pan-specific” flaviviral protease inhibitor in direct comparative analysis to the dengue virus NS3 protease. Previous reports in the literature have suggested differences in the mechanism of NS2B – cofactor dependent activation of the JEV and dengue protease. Homology modeling has suggested that residues Ala67–Asp76 of JEV NS2B provide additional interactions to stabilize the fold of the protease whereas residues Lys78–Leu87 of JEV NS2B are involved in the formation of the active site by an ‘induced fit’ mechanism

The NS2B/NS3pro protease of Japanese encephalitis virus was cloned in *E. coli*, overexpressed and purified. The sequence of NS2B/NS3 from JEV was obtained by custom-synthesis of the full-length gene and the active protease complex NS2B(H)/NS3 pro was generated by SOE-PCR. Protein was purified by metal affinity chromatography and size-exclusion FPLC and samples were analyzed by SDS-PAGE, Western immunoblotting and assay with 2 synthetic model substrates, RTKR-amc and GRR-amc. This analysis yielded – for the first time – kinetic parameters for K_m and k_{cat} of the JEV NS3 protease. The data also revealed only minor differences between the dengue virus NS3 protease and JEV NS3, although both enzymes had comparable requirements for pH and ionic strength. Activity of JEV NS2B(H)-

NS3pro appeared to be suboptimal under physiological conditions as the optimal pH was determined to 9.5 and a 50% inhibition of enzyme activity was observed at a relatively low concentration (12.5 mM) of NaCl.

II) ANALYSIS OF THE NS3 PROTEIN NTPase AND RNA HELICASE ACTIVITIES

At present, it still remains unclear whether the distinct enzymatic activities of the dengue virus NS3 protein (serine protease, NTPase and RNA helicase) are functionally independent or – as suggested recently by literature observations with the hepatitis C protease – whether their activities are modulated by an interplay of the domain elements. It was demonstrated earlier that the NS3 protease/helicase specifically interacts with 3' terminal sequences of the viral RNA and that the NTPase/helicase activity is modulated by the presence of polynucleotides whereby addition of polyU represses RNA helicase activity.

As a prelude to the investigation of domain-domain interactions within the NS3 protein, we have initiaed the cloning and analysis of the NS3 NTPase and RNA-stimulated helicase activities. Within this subproject, assays for both the NTPase and the RNA helicase had to be established.

We have – in close analogy to literature protocols - employed methods for the cloning, purification and assay of the dengue virus NS3 NTPase and RNA helicase. Plasmids harboring the NS3 helicase domain and the NS2B hydrophilic domain linked to the NS3 full-length gene, NS2B(H)NS3FL, were constructed by PCR and restriction enzyme digestion. The two recombinant proteins were expressed in *E. coli*. The 56.5 kDa NS3h was purified by metal chelate affinity chromatography followed by renaturation of precursors, mediated by artificial chaperone-assisted refolding, which yielded the active helicase. NTPase assay was based on colorimetric method with malachite green reagent. The NTPase activity of NS3 helicase in the presence of ATP showed a higher turnover and K_m value than without ATP. The activity

increased approximately 3-fold in the presence of polynucleotides. This indicates that NTPase activity of dengue NS3 can be stimulated by the addition of polynucleotides. With NS3h, a helicase assay was conducted using short internally quenched DNA oligonucleotides. Significant signal increase was observed in the presence of polynucleotides. No unwinding activity was observed with addition of poly U. These results suggest that the binding sites for polynucleotides and the RNA duplex on NS3 protein are essentially identical.

III) BIOCHEMICAL EVALUATION OF PROTEOCHEMOMETRIC MODELS FOR ENZYME – SUBSTRATE / INHIBITOR INTERACTION

In collaboration with Prof. Jarl E. S. Wikberg, Dept. of Pharmaceutical Biosciences, Uppsala University, Sweden, we have discovered and assayed a few extremely potent compounds generated by theoretical molecular design principles. The advantage of proteochemometrics (PCM) is that it can very efficiently generate structures for lead candidates with high-affinity inhibition on a relatively moderate set of input parameters such as sequence, active site topology and 3-dimensional structures. The best compound obtained so far has a K_i < 10 nm. We have considered patenting some of those compounds, however, we concluded that the available data are too preliminary to justify patenting efforts as we have little or no information about effects on viral replication in vivo, cytotoxicity, bioavailability etc. In collaboration with Prof. Jarl E. S. Wikberg, Dept. of Pharmaceutical Biosciences, Uppsala University, Sweden, we have completed a significant study on the development and refinement of proteochemometric models for the dengue virus NS3 protease (*Bioorg. Med. Chemistry (2008),* ***16****, pp. 9369-9377*). In particular, the prime side specificity of the dengue virus NS3 protease was investigated by a target interaction analysis using statistical molecular design (SMD). Analysis of the models derived from kinetic assays with 48 internally

quenched peptides demonstrated that different physico-chemical properties of the amino acid residues in the prime side region contributed independently to K_m and k_{cat} activities and this has also identified residues that are favorable for substrate affinity as well as cleavage efficiency, respectively. We are currently investigating structural predictions for dengue virus NS3 protease inhibitors by assay with a library of non-native peptide compounds which were custom-synthesized by our collaborator, Dr. Taian Cui, at Singapore Polytechnic College. The peptides were designed by proteochemometric modeling by our collaborator, Dr. Jarl Wikberg at Uppsala University, Sweden. Preliminary data will be useful for the design synthesis and evaluation of potential future protease inhibitors. It should be noted, that both groups in Sweden and Thailand receive funding for a collaborative research project from the Swedish International Development Cooperation Agency (SIDA), which is dedicated exclusively to the exchange of students and travel of the principal investigators, but does not cover expenses for equipment and chemicals. Currently, a PhD student from Prof. Wikberg's group (Mr. Muhammad Junaid) is working at IMB on screening of a synthetic inhibitor library with NS3 proteases from 4 dengue virus serotypes which would be useful to demonstrate the quality of predictive denominators for enzyme-ligand binding which have been developed so far by *in silico* methods. The ultimate goal of this project would be a joint PCM map for proteases of the flavivirus complex and a common route to inhibitors for structurally heterogeneous drug targets.

IV) COMPARISON OF BIOCHEMICAL PROPERTIES BETWEEN 4 DENGUE VIRUS SEROTYPES OF NS3 PROTEASES

We were able to demonstrate that despite possessing obvious similarities in structure and reactivity, the NS3 proteases from 4 dengue serotypes display a number of distinct properties such as expression yield

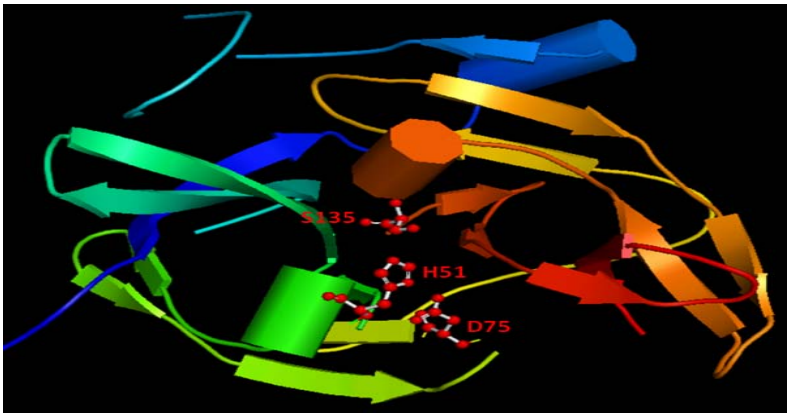
in *E. coli*, protein stability, refolding kinetics, susceptibility to proteolytic degradation and kinetic differences when assayed with model substrates for serine proteases such as GRR-amc. These investigations question the recently proposed concept of a “pan-specific” inhibitor which would be equally effective against all 4 dengue serotypes.

In the successfull M.Sc. thesis of Mr. Tawin Iempridee, enzymatically active forms of the protease complexes were constructed by RT-PCR and subsequent SOE-PCR. Recombinant proteins were expressed in *E. coli* and purified to homogeneity by metal chelate affinity chromatography followed by size-exclusion chromatography under denaturing conditions in the presence of 8 M urea. Renaturation of precursor enzymes was initiated by using an artificial chaperone-assisted refolding technique which basically mimicks the bacterial GroE system *in vitro*. Gentle refolding of the precursor protein(s) was achieved by using cyclic α-1,4-glucan (cycloamylose) as renaturing agent for protein-detergent complexes. For the first time, we have introduced active-site titration (using aprotinin as a tight binding inhibitor) and inner filter effect correction to obtain precise estimates for enzyme activity. Kinetic analysis revealed comparable K_m values for the enzymes, whereas k_{cat} values displayed much larger variations. Stability assays also demonstrated some unexpected differences in half-life at 50º C. However, all proteases shared similar pH optima and ionic strength dependence as well as overall tertiary structures as determined by fluorescence spectroscopy. These findings support the notion that genetic diversity of protease complexes among the 4 serotypes of dengue virus has led to marked differences in thermostability and proteolytic efficiency, while retaining an overall structural conservation.

It is conceivable that the differences we have observed contribute to disparate inhibition profiles towards a given protease inhibitor. It is not known at present whether these alterations contribute to the different kinetics for virus replication and maturation which were observed in a number of *in vitro* studies.

V) IDENTIFICATION OF KEY RESIDUES FOR SUBSTRATE BINDING BY STRUCTURE-GUIDED MUTAGENESIS

Within her successful M.Sc. thesis project, Mrs. Wanisa Salaemae has demonstrated that a number of residues between dengue virus and West Nile virus display noteable differences in their role for substrate binding and catalysis. We have generated by site-directed mutagenesis alanine substitutions of a number of conserved residues, L115, D129, G133, T134, Y150, G151, N152, S163 and I165 in the S1 and S2 subsites of the NS3 active site to probe the contribution of individual residues to substrate binding, catalysis and to transition state stabilization by formation of an oxyanion hole. Mutant enzymes were probed by fluorescence release from the model substrate GRR-amc. We found that mutants Y150A and G151A had completely abrogated enzyme activity, whereas a G133A mutant displayed approx. 10-fold reduction in activity when compared to the wild-type, possibly due to a contribution of this residue to the formation of catalysis-competent oxyanion hole. Although essentially in agreement with earlier data for the West Nile virus NBS3 protease, we found marked differences in catalytic efficiency for a number of positions thus suggesting a distinctive organization of the dengue virus NS3 protease active site. This study described for the first time the effect of active site mutations in the dengue virus NS3 protease by a sensitive assay using a fluorescent substrate supplied for *in trans* cleavage reactions.



Structure of the dengue virus NS3 protease active site.

OUTPUT

Publications:

1. Salaemae, W., Junaid, M., Angsuthanasombat, C. and **Katzenmeier, G.** (2010). Structure-guided mutagenesis of active site residues in the dengue virus two-component protease NS2B-NS3. *J. Biomed. Sci.* **17**, 68-75. (**impact factor 2.01**)

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3. Iempridee, T., Thongphung, R., Angsuthanasombat, C. and **Katzenmeier, G.** (2008). A comparative biochemical analysis of the NS2B(H)-NS3pro protease complex from four dengue virus serotypes. *Biochim. Biophys*. *Acta*, **1780**, 989-994. (**impact factor 2.96**)

Conferences:

Tongphung, R. and **Katzenmeier, G.** (2007). Cloning, expression and purification of the dengue virus NS3 helicase domain and analysis of catalytic properties. 1st Biochemistry and Molecular Biology (BMB) Conference: Biochemistry and Molecular Biology for the Integration of Life. Bangkok, Thailand, April 26-27.

Tongphung, R. and **Katzenmeier, G.** (2007). Cloning, expression and purification of the dengue virus NS3 helicase/NTPase domain and analysis of catalytic properties. 8th National Grad Research Conference. Mahidol University, Salaya, Thailand, September 7-8.

Chalayut, C. and **Katzenmeier, G.** (2007). Enzymological characterization of the NS2B(H)/NS3 protease of Japanese encephalitis virus. 2nd Annual Symposium of Protein Society of Thailand. Chulabhorn Research Institute Conference Center, Bangkok, Thailand, September 20-21.

Tongphung, R. and **Katzenmeier, G.** (2007). Expression, purification and characterization of the helicase/NTPase activity of dengue virus type 2 NS3 helicase domain. 2nd Annual Symposium of Protein Society of Thailand. Chulabhorn Research Institute Conference Center, Bangkok, Thailand, September 20-21.

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Katzenmeier, G. (2008). Dengue virus nonstructural proteins NS2B/NS3 – molecular targets for drug discovery. Drug Design and Discovery for Developing Countries. *Invited speaker at* International Centre for Science and High Technology of the United Nations Industrial Development Organization (ICS – UNIDO). Trieste, Italy, July 3-5.

Katzenmeier, G. (2008). A novel therapeutic target against dengue virus diseases – the NS2B/NS3 two-component protease. *Invited speaker at* 3rd Annual Symposium of Protein Society of Thailand. Chulabhorn Research Institute Conference Center, Bangkok, Thailand, August 28-29.

Thongphung, R. and **Katzenmeier, G.** (2008). Biochemical analysis of the dengue virus NS3 protease / RNA helicase enzymatic functions. 3rd Annual Symposium of Protein Society of Thailand. Chulabhorn Research Institute Conference Center, Bangkok, Thailand, August 28-29.

Chalayut, C. and **Katzenmeier, G.** (2008). Enzymological studies on the Japanese encephalitis virus NS2B/NS3 serine protease. 3rd Annual Symposium of Protein Society of Thailand. Chulabhorn Research Institute Conference Center, Bangkok, Thailand, August 28-29.

Thongphung, R. and **Katzenmeier, G.** (2008). Functional interdependence of the dengue virus NS3 serine protease and NTPase / helicase domains. The 2nd International Conference on Dengue and Dengue Haemorrhagic Fever. Phuket, Thailand, October 15-17.

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Wikberg, J. E. S., Lapins, M., Prusis, P., Yahorava, S., Petrovska, R., Niyomrattanakit, P. and **Katzenmeier, G.** (2008). Design of inhibitors for dengue virus NS3 proteases by use of proteochemometrics. The 2nd International Conference on Dengue and Dengue Haemorrhagic Fever. Phuket, Thailand, October 15-17.

Chalayut, C. and **Katzenmeier, G.** (2009). Characterization of the two-component NS2B(H)-NS3 protease of Japanese encephalitis virus. 4th Annual Symposium of Protein Society of Thailand. Chulabhorn Research Institute Conference Center, Bangkok, Thailand, August 26-28.

Junaid, M. (2010). Structure-guided mutagenesis of active site residues in the dengue virus two-component protease NS2B-NS3. (*oral presentation*) 1st NCOBAM Conference of Biotechnology and Microbiology, Bara Gali, Pakistan, July 4-7.

Salaemae, W., Junaid, M., Angsuthanasombat, C. and **Katzenmeier, G.** (2010). Identification of key residues for selective binding and catalysis of the dengue virus NS2B-NS3 protease. Joint International Tropical Medicine Meeting (JITMM), Bangkok, Thailand, December 1-3.

Muhammad Junaid, Wanisa Salaemae, Chanan Angsuthanasombat, Jarl Wikberg and **Gerd Katzenmeier** (2011). (*oral presentation*) Identification of key determinants of selective binding and catalysis in the active site of dengue virus NS3 protease. Annual Scientific Meeting of the Australian Society for Microbiology, Hobart, Australia, July 4-8.

STUDENTS GRADUATED BY THESIS WORK IN THE PROJECT:

(it is noteworthy that this project has been performed with only 2 MSc students as listed below)

NAME	DATE OF GRADUATION	THESIS TITLE
MSc students:		
Ratchanu Tongphung	April 29, 2009	Cloning, expression and purification of the dengue virus NS3 helicase domain and analysis of the catalytic properties
Chakard Chalayut	December 9, 2009	Cloning, expression, purification and enzymological characterization of NS2B/NS3 protease protein of Japanese encephalitis virus