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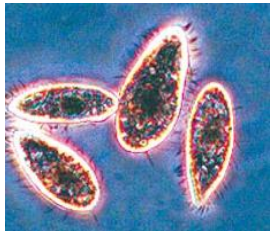
Multipotent Natural Leads against Respiratory Tract Infections

Judith M. Rollinger
Chair of Phytochemistry & Biodiscovery
Department of Pharmacognosy
Faculty of Life Science, University of Vienna
e-mail judith.rollinger@univie.ac.at

Department of Pharmacognosy

Plants for Health, Zurich, 01.12.2016

Why natural products?



Natural products: "***privileged structures***"

(Koch & Waldmann, In: Methods and Principles in Medicinal Chemistry, Wiley-VHC, 2004)

- complex chemical scaffolds (Feher & Schmidt, JCIM 2003 43, 218–27)
- high chemical & biological diversity (Rosen et al. , J. Med. Chem. 2009 52, 1953–62)
- ..result of millennia of selection for self defense and signaling
- many untapped natural sources (microorg., algae, fungi, marine org., ..)

> 50 % of drugs can be traced to or were inspired by **natural products**

Newman D.J. and Cragg G.M. (2012) J. Nat. Prod., 75, 311-335

How to best access the natural products' bioactivity?

https://www.aspectra.ch/blog-und-news/know-how/die-nadel-im-heuhaufen---5-tipps-wie-sie-in-logfiles-schnell-informationen-finden/

Information ....

- 300.000-500.000 species of plants ; total no of sp.: ~2 mill. (Hammond, 1995)
- >270.000 natural products (Dict. of Nat. Prod.)
- ~85.000 protein structures (www.rcsb.org/pdb/)
- ~3.000-10.000 druggable targets;
- ~500 curr. exploited (Overington et al. 2006)

→ **Data mining**

Complementary!! ↕ **Synergistic?**

Ethnopharmacology

Records about beneficial effects of herbal remedies /traditional medicine

→ what's behind: proven efficacy, low toxicity - placebo, anecdotal lores?

In silico strategy in NP research

- Predictions are only as good as the input information!
- Reliability of the in silico tool! → Validation!
- Enrichment factor – hit rate!
- Successful predictions: ~10-20 %

Virtual Screening → Data Mining

→ Further selection criteria are necessary/advisable

- → *in vitro* testing of extracts
- → ethnopharmacological hints
- → phenomenological effects
- → activity profiling

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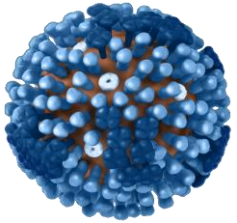
Resistance breaking inhibitors of influenza neuraminidase



FWF project: *Natural products targeting influenza*

Aim: Identification of novel anti-influenza agents

→ to overcome resistance and

→ to develop multidrug strategies

Virology group
(University of Jena)
Heike Braun
Elisabeth Walther
Michaela Schmidtke

Universitätsklinikum Jena

Pharmacognosy group
(University of Vienna)
Christina Mair
Ulrike Grienke
Judith M. Rollinger

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Theoretical Chemistry group
(University of Innsbruck)
Susanne von Grafenstein
Klaus Liedl

universität innsbruck

Johannes Kirchmair
(University of Hamburg)

Influenza A (H1N1 & H3N2) and B viruses

Incubation time: 12 - 72 h

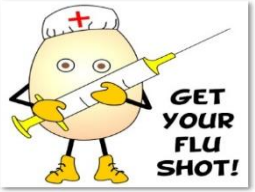
Symptoms:

picture: www.loohealth.com; avalonlibrary.org

morbidity and mortality: ~ 500,000/year

high among infants, elderly, and in immunocompromised

prevention → vaccination:

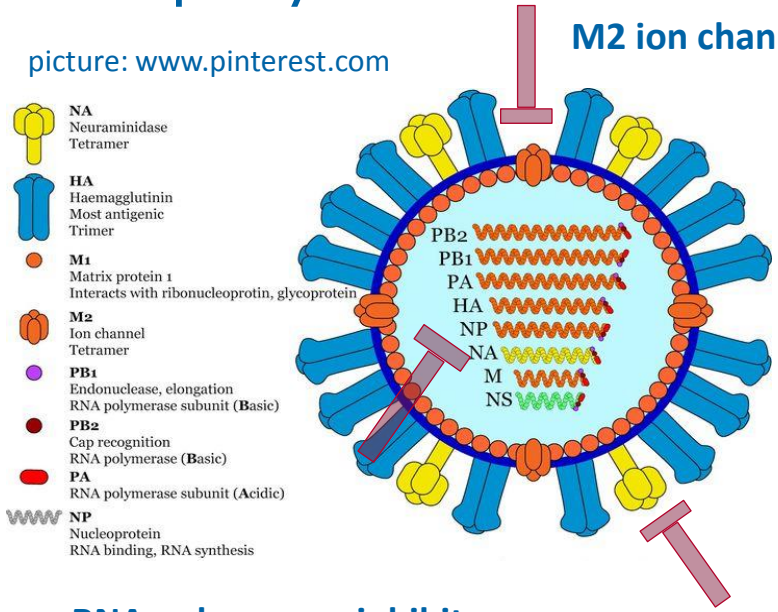


trivalent or quadrivalent vaccine: A/H1N1, A/H3N2, B (B), annually

efficacy: 70 - 90% in young adults, ~50% in elderly, 3 to 6 months

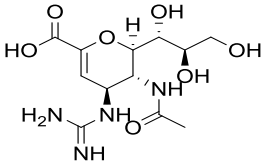
Prophylactic & therapeutic agents

picture: www.pinterest.com

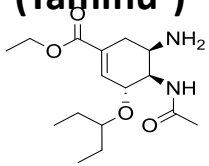


since 2009 not recommended → resistance

zanamivir (Relenza®)

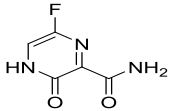


oseltamivir (Tamiflu®)



RNA polymerase inhibitors

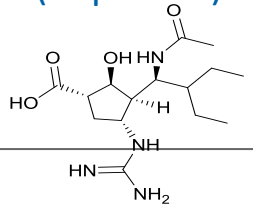
favipiravir (T-705; Avigan®)



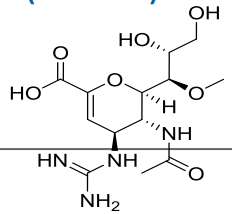
approved for treating novel or resistant flu infections in Japan in 2014 (good tolerance)

neuraminidase inhibitors (NAIs)

peramivir (Rapiacta®)



laninamivir (Inavir®)



Furuta, et al. - *Antivir. Res.* 2013

NA inhibitors from nature

Cite this: *Nat. Prod. Rep.*, 2012, **29**, 11

www.rsc.org/npr

REVIEW

Influenza neuraminidase: A druggable target for natural products

Ulrike Grienke,[a] Michaela Schmidtke,[b] Susanne von Grafenstein,[c] Johannes Kirchmair,[d] Klaus R. Liedl[c] and Judith M. Rollinger*[a]

Received 4th July 2011

DOI: 10.1039/c1np00053e

1 Introduction
2 Influenza NA – an established drug target for combating influenza
2.1 Influenza NA function
2.2 Influenza NA classification
2.3 Influenza NA structure
2.4 Catalytic mechanism
2.5 Approved NAIs
2.6 Mechanisms conferring drug resistances in influenza
2.7 Similarities and differences of influenza NA with NA of other biological systems
3 Experimental evaluation of NA inhibition
3.1 Methods used in discovery and functional analysis of first generation NAIs
3.2 Advantages and disadvantages of methods currently applied to identify novel NAIs
4 Inhibition of influenza NA by natural compounds
4.1 Evaluation of published data
4.2 Compound classes and sources of natural NAIs
4.3 Phenolic compounds as NAIs
4.4 Isoprenoids – a compound class showing no effect on NA
4.5 Extracts with promising NA inhibitory activity
5 Strategies for the discovery of NA inhibiting natural compounds
5.1 *In vitro* screening of extracts–Bioassay-guided fractionation and isolation
5.2 Exploitation of empirical knowledge
5.3 Exploiting influenza NA inhibition by computer-guided approaches
6 Conclusions and future perspectives
7 References

[a] *Institute of Pharmacy/Pharmacognosy and Center for Molecular Biosciences Innsbruck, University of Innsbruck, Innrain 52c, 6020 Innsbruck, Austria. E-mail: Judith.Rollinger@uibk.ac.at*
[b] *Department of Virology and Antiviral Therapy, Jena University Hospital, Hans-Knoell-Straße 2, 07745 Jena, Germany*
[c] *Institute of Theoretical Chemistry and Center for Molecular Biosciences, University of Innsbruck, Innrain 52a, 6020 Innsbruck, Austria*
[d] *Unilever Centre for Molecular Informatics, Department of Chemistry, Lensfield Road, Cambridge, UK CB2 1EW*

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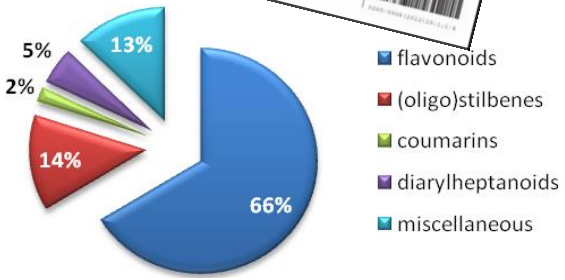
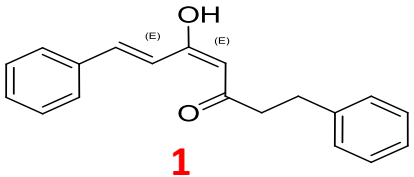


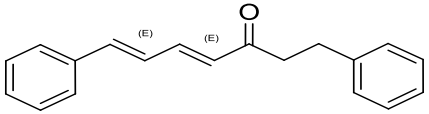
Percentage of natural product cpd classes showing inhibition of influenza NA (from 2000 - 11)

NA inhibitors from *Alpinia katsumdai*



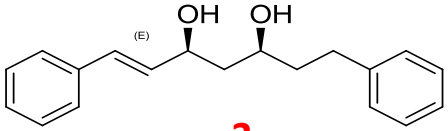
1

(E,E)-5-hydroxy-1,7-diphenyl-4,6-heptadien-3-one



2

(E,E)-1,7-diphenyl-4,6-heptadien-3-one



3

(3S,5S)-trans-3,5-dihydroxy-1,7-diphenyl-1-heptene

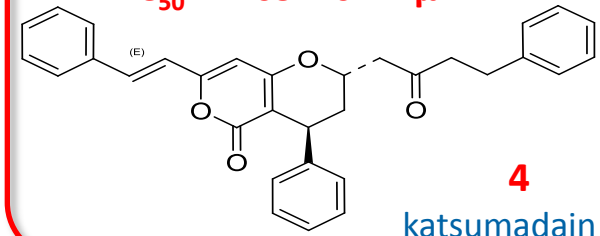
Seeds used in TCM: antiviral herbal remedy

ingredient of complex Chinese antiviral preparations

unknown molecular mechanism

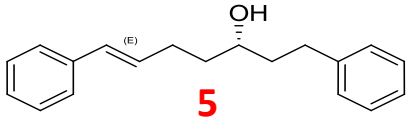
unknown bioactive constituents

IC_{50} = 1.05 ± 0.42 µM



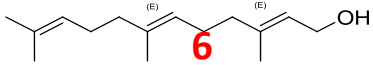
4

katsumadain A



5

(S)-1,7-diphenyl-6(E)-hepten-3-ol



6

trans,trans-farnesol

Grienke. et al., *J. Med Chem*. 2010, 53, 778-86

In silico rationalization

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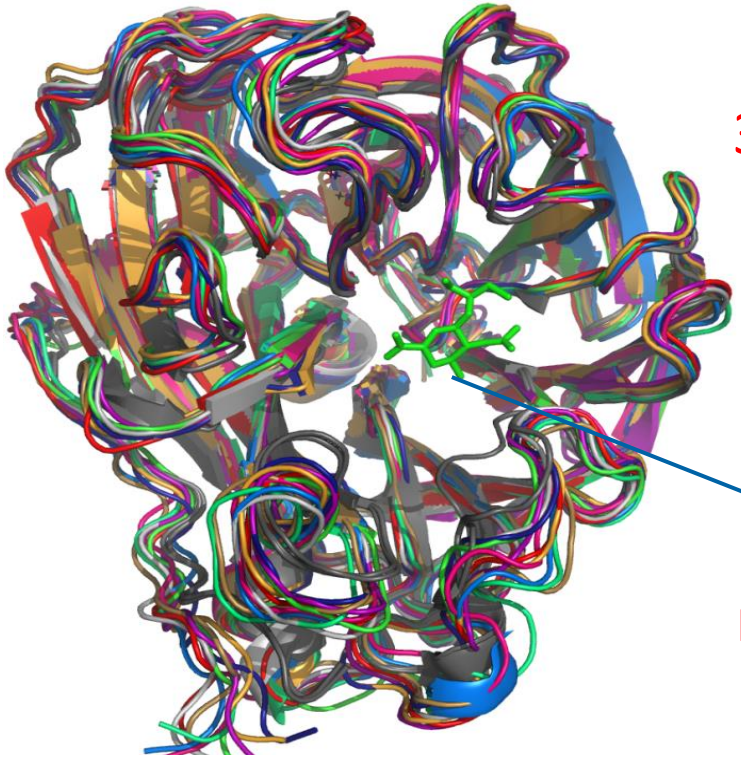

Oseltamivir

3 NA X-ray structures used for docking

green color: closed conformation (2hu4)

red color: open apo-structure (2hty)
→ macromolecule without ligand bound

blue color: open holo-structure (2hu0)
→macromolecule with its ligand bound

Molecular dynamic simulations (AMBER 10.0)

Computational studies → conformational flexibility in 150- & 430-loop *
Conformational shifts → accommodate novel, bulkier and more potent binders

* Amaro R.E. et al. *J. Am. Chem. Soc*. 2007,129, 7764

778 *J. Med. Chem.* **2010,** *53,* 778–786
DOI: 10.1021/jm901440f

Journal of Medicinal Chemistry
Article

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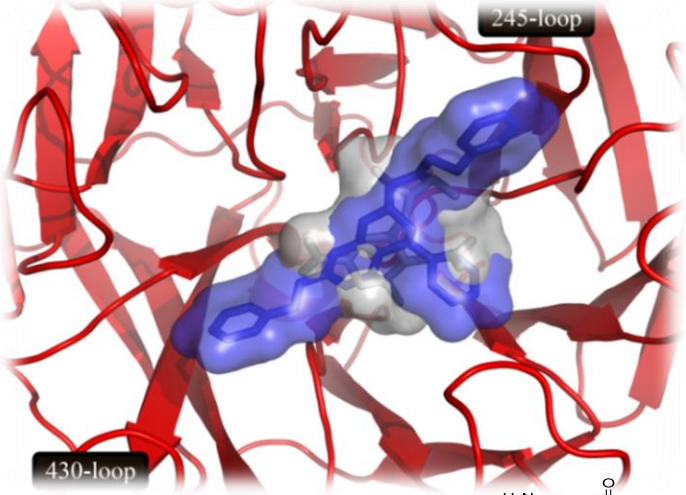
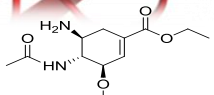
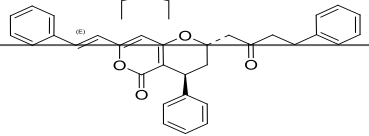
Antiviral Potential and Molecular Insight into Neuraminidase Inhibiting Diarylheptanoids from *Alpinia katsumadai*

Ulrike Grienke,[†] Michaela Schmidtke,[‡] Johannes Kirchmair,[§] Kathrin Pfarr,[‡] Peter Wutzler,[‡] Ralf Dürrwald,[⊥] Gerhard Wolber,[§] Klaus R. Liedl,[∥] Hermann Stuppner,[†] and Judith M. Rollinger*,[†]



Oseltamivir (gray)





Katsumadain A (blue)

1. Rationalizing the putative interaction of katsumadain A in the flexible NA binding site

2. Binding hypothesis in accordance with experimentally observed activity variations of wild type and mutant NA

→ **Katsumadain A = novel iNAI-template**

→ **Confirmation of working hypothesis?**

Mining the chemical space of NAI

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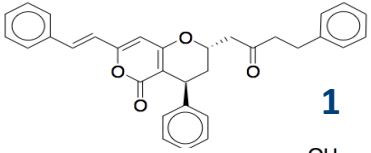
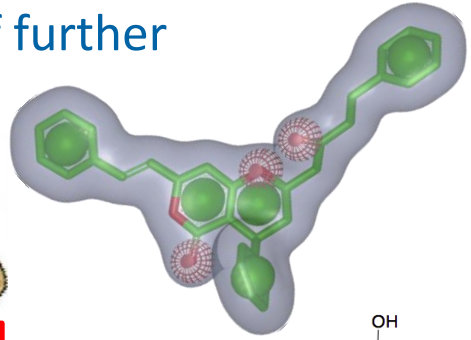
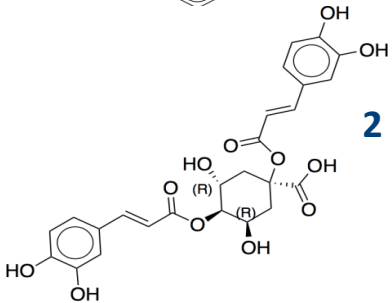
Shape-focused virtual screening and identification of further natural hit compounds → 5 compounds selected



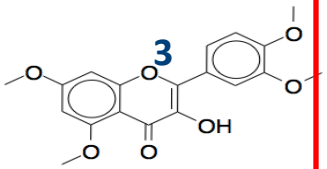
1 katsumadain A



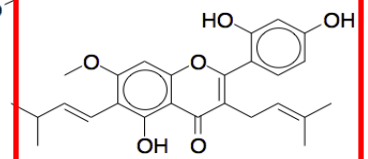
ROCS Vers 3.0.0
OpenEye, NM, USA



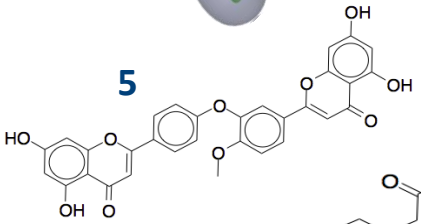
2 dicaffeoylquinic acid
IC_{50} = 17.0 µM



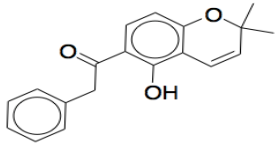
3 quercetin deriv.
1.09 µM



4 artocarpin
0.18 µM



5 biflavone
2.05 µM



6 2H chromen deriv.
1.33 µM

Determination of their NA-inhibiting potential using a chemiluminescence based assay for NA inhibition (PR/8/34)

Kirchmair J. et al. *Future Med Chem* 2011, 3, 437-50

Antiviral profiling

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Cytototoxicity and antiviral activity of katsumadain A (1) and five virtually predicted test compounds (2-6)

Compound	CC_{50} in MDCK cells (µM)	50% NA-inhibitory conc. (µM) determined for influenza virus A/			
		oseltamivir-suscept. strains			oseltamivir-resist.
		PR/8/34	Jena/5528/09	Jena/5555/09	342/09
1	88.66	2.14	1.76	1.72	4.55
2	>193.61	17.0	nd	nd	nd
3	>279.33	1.09	0.39	0.98	14.83
4 Artocarpin	19.43	0.18	0.23	0.30	0.55
5	>180,83	2.05	3.54	2.01	40.65
6	21.78	1.33	2.04	2.21	6.35
Oseltamivir carboxylate		0.23	0.12	0.13	>30 nM

Mean dose response curves (n=3)

Kirchmair J. et al. *Future Med Chem* 2011, 3, 437-50

Exploration of the NA binding site

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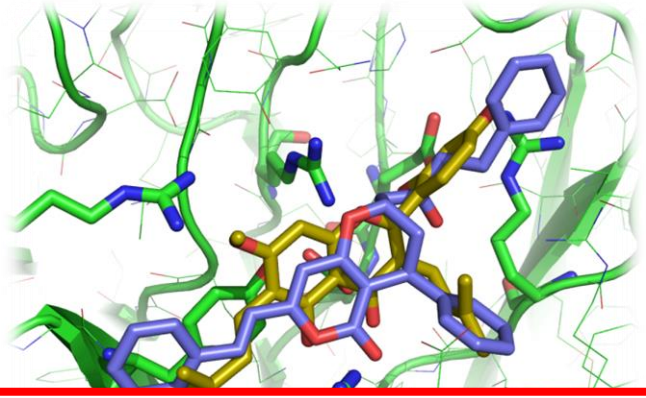


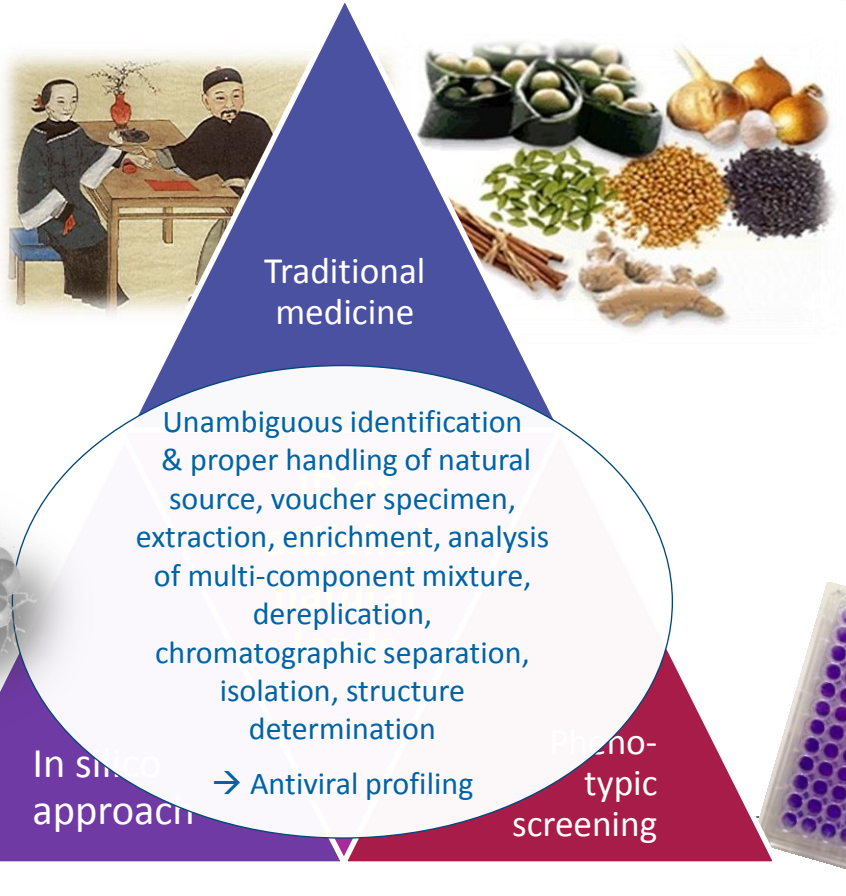
Overlay of docking poses (GOLD) of katsumadain A (violet) & artocarpin (yellow) in the open frame

→ Starting point for the identification of new resistance-breaking synthetic NA inhibitors

Kirchmair, J.; Schmidtke, M.; Liedl, K.; Rollinger, J.M. (2013): **Compounds for the treatment of influenza.**

EP 2599480 A1 20130605

Combined approach in NP research



Combined approach in NP research

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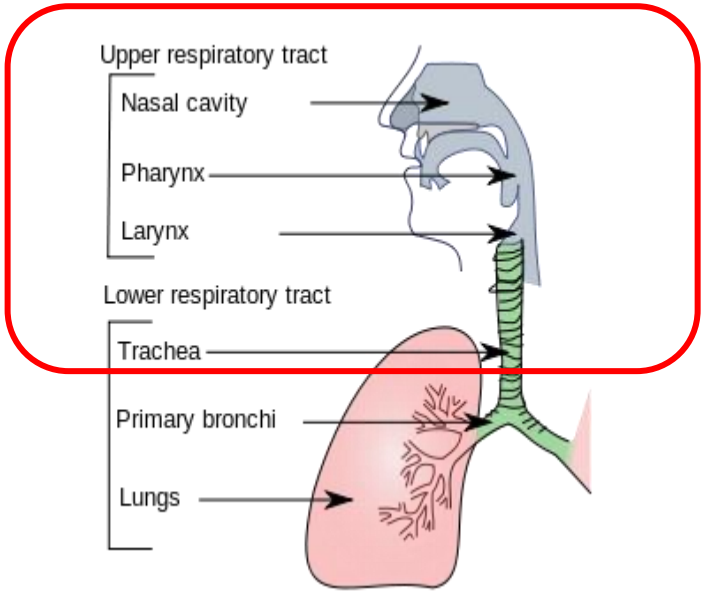
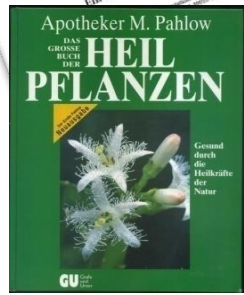
Identification of antiviral natural cpds against upper respiratory tract infections (URTI)



Keywords in trad. medicine

- Fever
- Cough
- Headache
- Common cold
- Bronchitis
- Lung diseases

Plant selection based on traditional knowledge



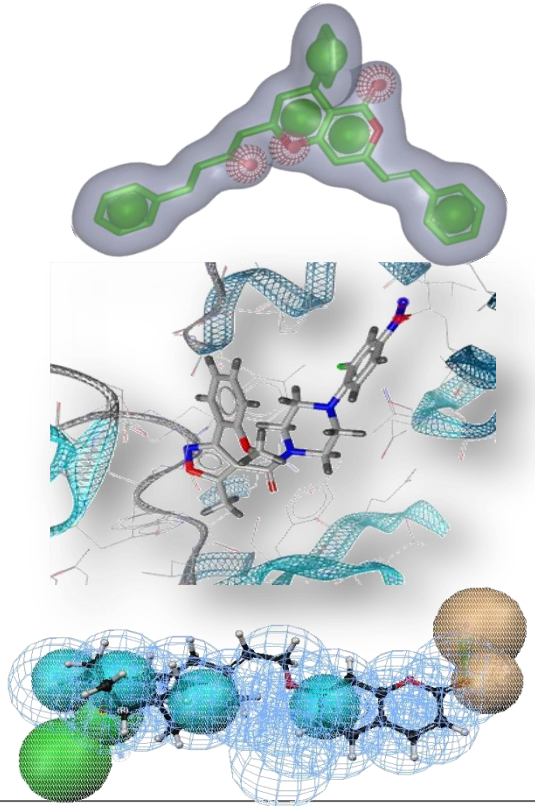
Knoblauch	*Allium sativum var. sativum*
Zimtapfel	*Annona squamosa*
Bärentraube	*Arctostapylos uvae ursi*
Wermut, Absinth	*Artemisia absinthum*
Beifuß, Wilder Wermut	*Artemisia vulgaris*
Wald-Bergminze	*Calamintha menthifolia*
Hirtentäschel, Bauernsenf	*Capsella bursa-pastoris*
Eberwurz	*Carlina acaulis*
Kümmel	*Carum carvi*
Edelkastanie	*Castanea vesca/ sativa*
Isländisch Moos	*Cetraria islandica*
(Wilde) Karotte	*Daucus carota*
Ackerschachtelhalm	*Equisetum arvense*
Augentrost	*Euphrasia officinalis ssp. rostkoviana*
Buchweizen	*Fagopyrum esculentum*
Fenchel	*Foeniculum vulgare*
Gemeiner Hohlzahn	*Galeopsis tetrahit*
Glänzender Lackporling	*Ganoderma lucidum*
Gundelrebe	*Glechoma hederaceae*
Süßholz	*Glycyrrhiza glabra*
Efeu	*Hedera helix*
Sonnenblume	*Helianthus annuus*
Leberblümchen	*Hepatica nobilis*
Chaga Pilz	*Inonotus obliquus*
Styrax, Amberbaum	*Liquidambar orientalis*
Bärlapp	*Lycopodium clavatum*
Kamille	*Matricaria chamomilla*
Zitronenmelisse	*Melissa officinalis*
Indische Lotosblume	*Nelumbo nucifera*
Wilder Majoran	*Origanum vulgare*
Klatschmohn	*Papaver rhoeas*
Schlafmohn	*Papaver somniferum*
Meisterwurz	*Peucedanum ostruthium*

Anis	*Pimpinella anisum*
Große Bibernelle	*Pimpinella major*
Fettkraut	*Pinguicula vulgaris*
Schwarzer Pfeffer	*Piper nigrum*
Spitzwegerich	*Plantago lanceolata*
Klapperschlangenwurzel	*Polygala senega*
Kreuzkraut	*Polygala vulgaris*
Engelsüß	*Polypodium vulgare*
Gänsefingerkraut	*Potentilla anserinae*
Großblütige Braunelle	*Prunella grandiflora*
Schwarze Johannisbeere	*Ribes nigrum*
Hagebutte	*Rosa canina*
Rosmarin	*Rosmarinus officinalis*
Brombeere	*Rubus fruticosus*
Weinraute	*Ruta graveolens*
Klebriger Salbei	*Salvia glutinosa*
Holunder	*Sambucus nigra*
Braunwurz	*Scrophularia nodosa*
Bittersüßer Nachtschatten	*Solanum dulcamara*
Echter Ziest	*Stachys officinalis*
Wald- Ziest	*Stachys sylvatica*
Storaxbaum	*Styrax calamitus*
Gamander	*Teucrium chamaedrys*
Quendel	*Thymus pulegioides*
Linde	*Tilia cordata / T. platyphyllos*
Huflattich	*Tussilago farfara*
Preiselbeere	*Vaccinium vitis-ideae*
Baldrian	*Valeriana officinalis*
Großblütige Königskerze	*Verbascum densiflorum*
Eisenkraut	*Verbena officinalis*
Ehrenpreis	*Veronica officinalis*
Wohlriechendes Veilchen	*Viola odorata*
Stiefmütterchen	*Viola tricolor*

Plant selection based on *in silico* approach

Focus on 3 antiviral targets

- **Influenza neuraminidase (NA)**
- **Influenza nucleoprotein (NP)**
- **Human rhinovirus capsid protein 1 HRV**



Grienke, U, et al, J. Med. Chem. 2010, 53(2), 778-786

Gerritz S.W., et al. PNAS 2011, 108,15366-71

Rollinger JM et al., J. Med. Chem. 2008, 51, 842-851

Antiviral testing

CPE inhibition assay on selected URTI viruses

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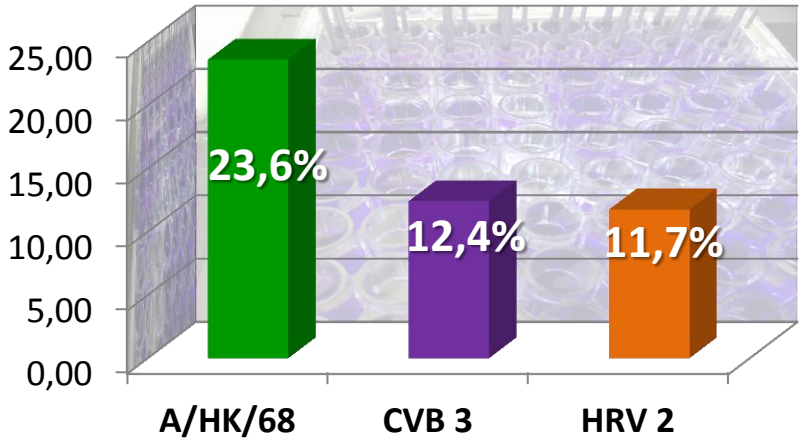
Percentages of active extracts (IC_{50}<50µg/mL)

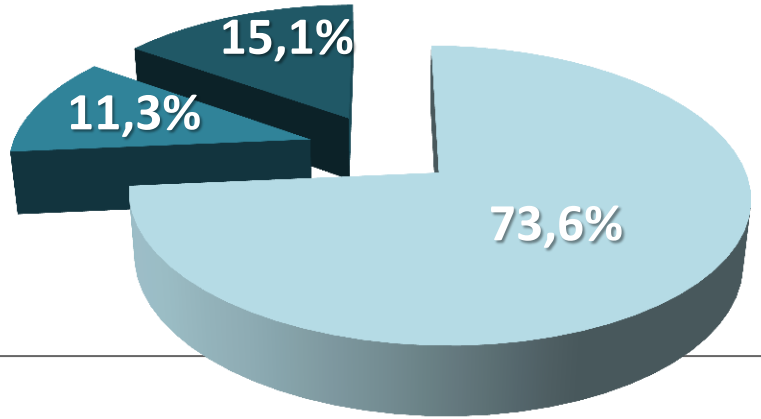


n = 161 for Infl. A/HK/68 (MDCK cells)

n = 153 for CVB3 & HRV3 (HeLa cells)

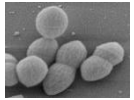
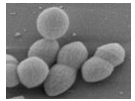
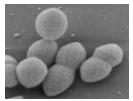
% of extracts (n=53)

- active against 1
- active against 2
- active against 3 viruses



Lethal interplay: viruses & bacteria

Co-pathogenesis of influenza viruses with bacteria in the lung:

1918/19	1957/58	1968/69	2009
H1N1 „Spanish flu“	H2N2 „Asian flu“	H3N2 „Hong Kong flu“	
~95% bacterial pneumonia 	most commonly *S. aureus*	?? 	30 - 50% 

Severe complications: by superinfections with *Streptococcus pneumoniae*

NAs present on both pathogens:

influenza NA: viral release & spread & attachment to receptors & nutrients for *S. pneumoniae*

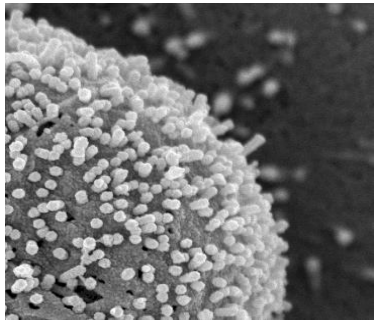
***S. pneumoniae* NA**: colonization, biofilm formation, and inflammation & rescue viral replication from inhibition by zanamivir

→ similarities in the NA active site conformation

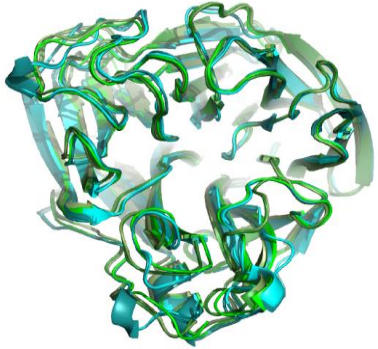
McCullers - *Nat. Rev. Microbiol.* 2014
John & Shanks - *Emerging Infect. Dis.* 2008

How to prevent the lethal synergism?

influenza virus

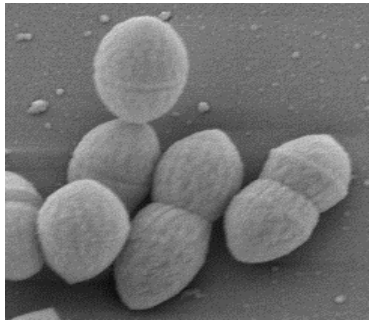


1 µm

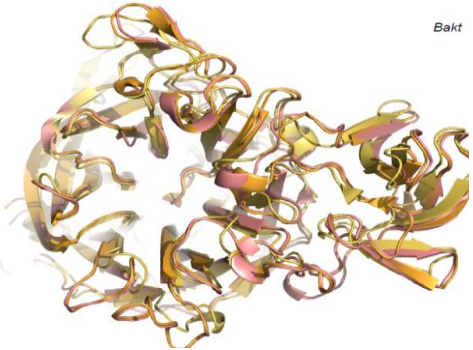


lethal synergism

pneumococci



1 µm



search for compounds which inhibit both pathogens

one target protein:

neuraminidase (NA)

N1 pH1N1 Zana bound 3b7e	N1 pH1N1 Apo open 3beq	N1 A/PR/8/34 Apo open Model	N2 HK/67 Bana bound 1ing	N2 HK/67 Sia bound 2bat

von Grafenstein, et al. *J. Biomol. Struct. Dyn.* 2015

Clostridium perfringens Dana bound, 2vk6	Streptococcus pneumoniae Apo, 2ya4	Streptococcus pneumoniae Zana bound, 2ya7

Follow-up study: *Morus alba* L.

Morus alba root bark: famous traditional medicine - Chinese Pharmacopoeia: sāng bái pí

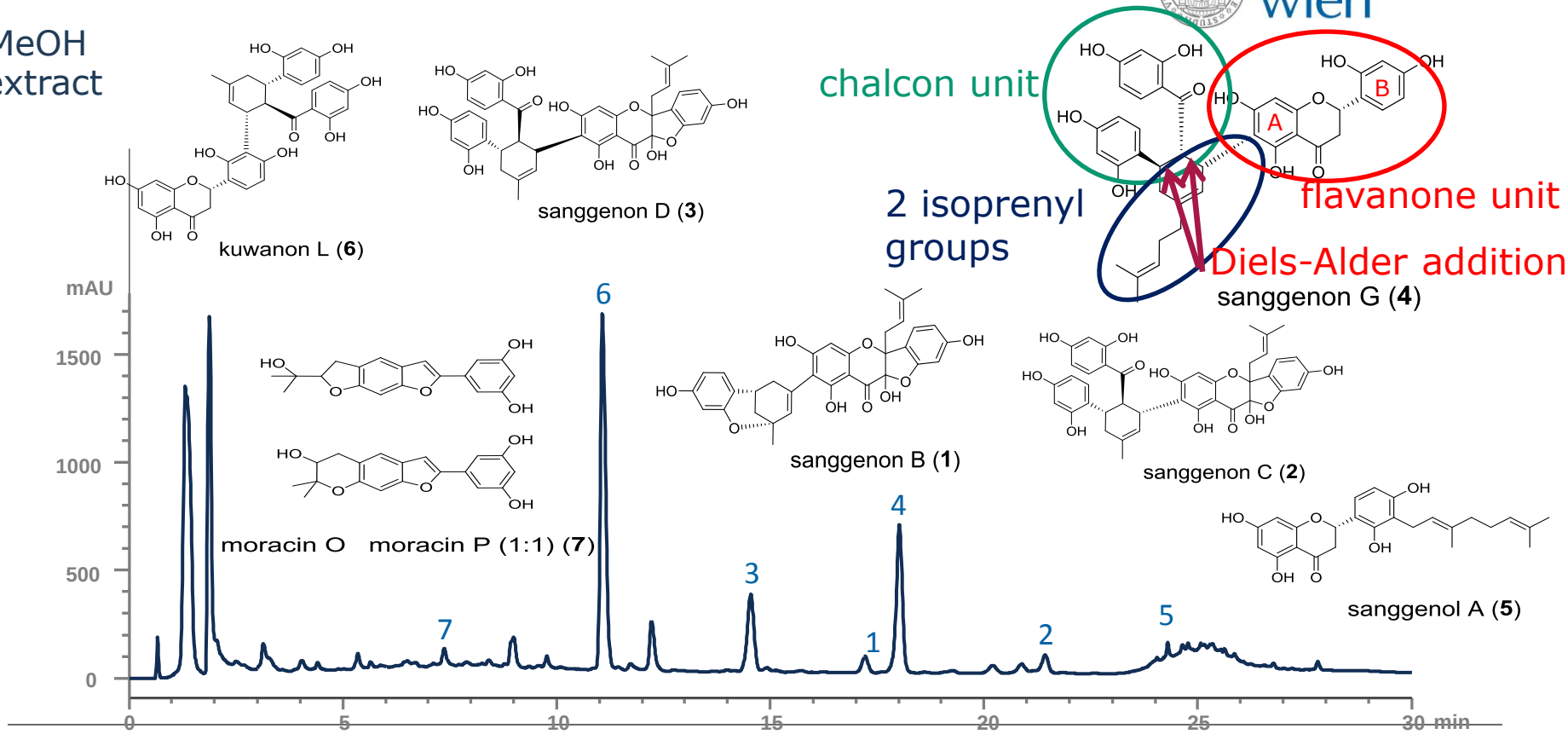
Ethnopharmcological use:

- against tonsillitis,
- antibacterial,
- against cough,
- asthma, pain,
- mucolytic

→ Moraceae rich in prenylated compounds

Modern translation of Alan Peck's Chinese Herbs

Phytochemistry of sāng bái pí



LC-parameter: stationary phase: Agilent Zorbax SB-C18 3.5µm (150 x 4.6 mm); temperature: 25 °C; mobile phase: A = water; B = acetonitrile; flow rate 1.0 mL/min; PDA detection wavelength: 205 nm; injection volume: 10 µL; gradient: 30% B; 5 min 45% B; 20 min 55% B; 22 min 98% B.

Assays: antiviral & antibacterial activity

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target-based

Hemagglutination (**HA**)-based NA inhibition **assay**

Fluorescence (**FL**)-based NA inhibition **assay**

pathogen-based

Inhibition of bacterial **biofilm**

Inhibition of bacterial **growth**

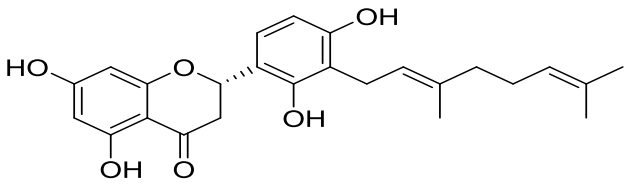
Cytopathic effect inhibition assay

Plaque-reduction assay

Richter, et al. – *Fut. Virol.* 2015

Antimicrobial activities [IC_{50} & MIC in µM]

Cpd	NA FL influenza 8178/09	NA FL pneumo-coccus	NA HA influenza 8178/09	NA HA pneumo-coccus	CC_{50} MDCK	CC_{50} A549 cells	90% MIC pneumococcus	90% MBIC pneumococcus
1	>100	15.0	>31.6	31.6	28.1	32.0	35.2	11.5
2	50.6	11.3	>3.16	>31.6	51.7	32.6	2.73	1.08
3	>100	[illegible]	[illegible]	[illegible]	[illegible]	79.9	8.72	5.89
4	30.9	[illegible]	[illegible]	[illegible]	[illegible]	72.9	5.81	1.55
5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	>100	5.05	4.44
6	>100	8.34	10	31.6	>100	94.4	48.5	23.0
7	>1	>1	77.2	100	80.8	>100	>50.0	>50.0



sanggenol A (**5**)

Congeners of sanggenol A (5)

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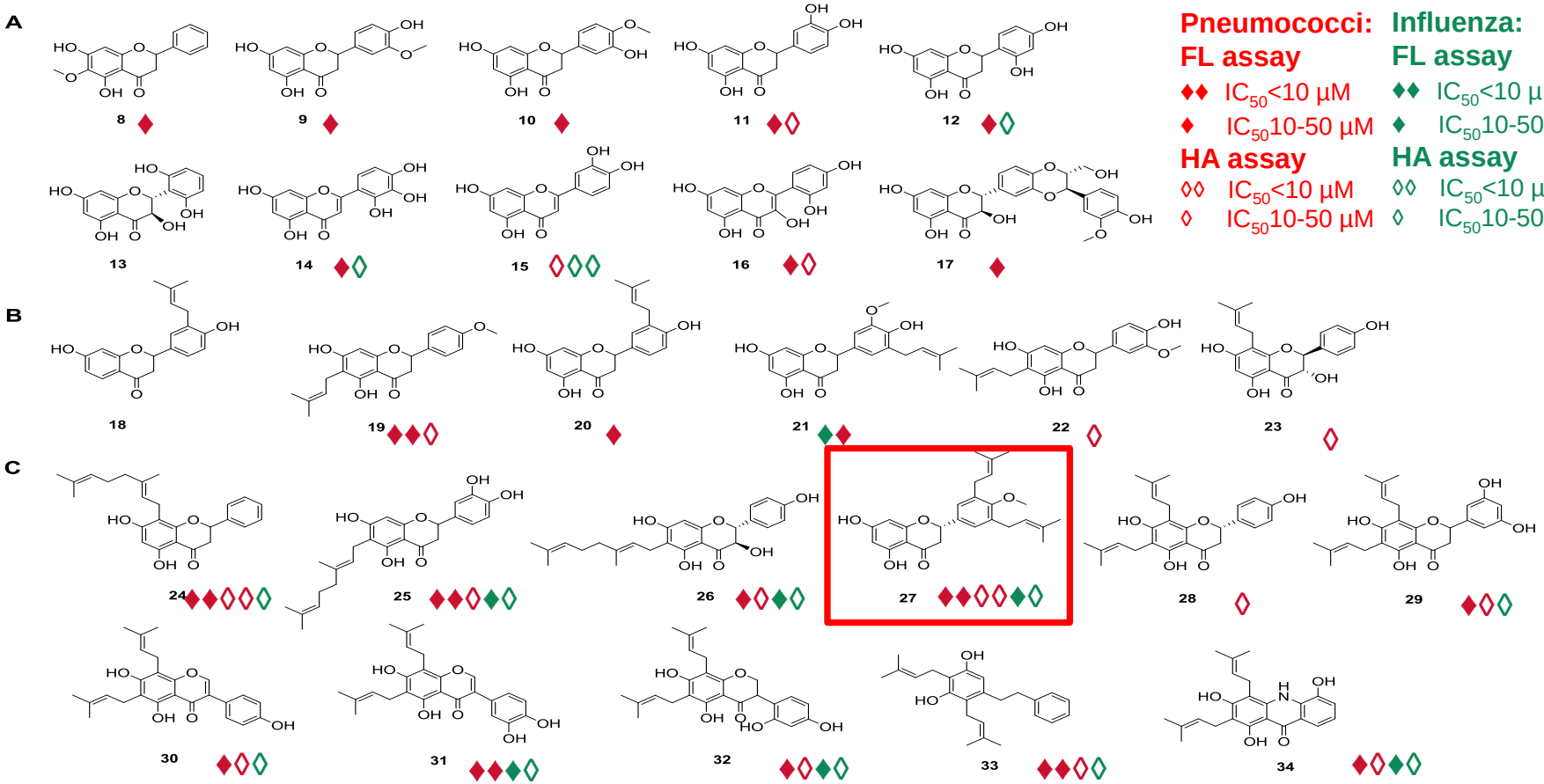
non-prenyl.

mono-prenyl.

di-prenyl.



Pneumococci:

FL assay

- ♦♦ IC_{50}<10 µM
- ♦ IC_{50}10-50 µM

HA assay

- ◊◊ IC_{50}<10 µM
- ◊ IC_{50}10-50 µM

Influenza:

FL assay

- ♦♦ IC_{50}<10 µM
- ♦ IC_{50}10-50 µM

HA assay

- ◊◊ IC_{50}<10 µM
- ◊ IC_{50}10-50 µM

Insights into ligand - target interactions?

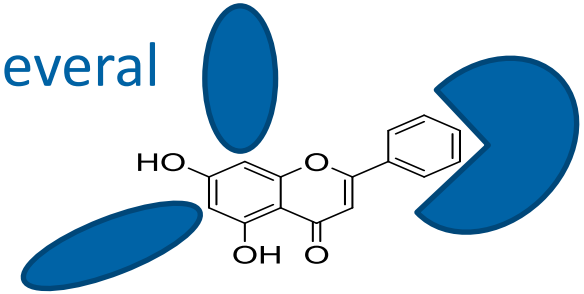
activity increases with degree of prenylation (explored in phenotypic and target-based assays)

activity increases with hydrophobicity

stronger bacterial NA inhibitory activities

alignment of bioactive congeners on flavonoid scaffold →

bulky substituents may be attached to several positions without loss of bioactivity



docking experiments support different ligand-binding possibilities!

Electron microscopy studies

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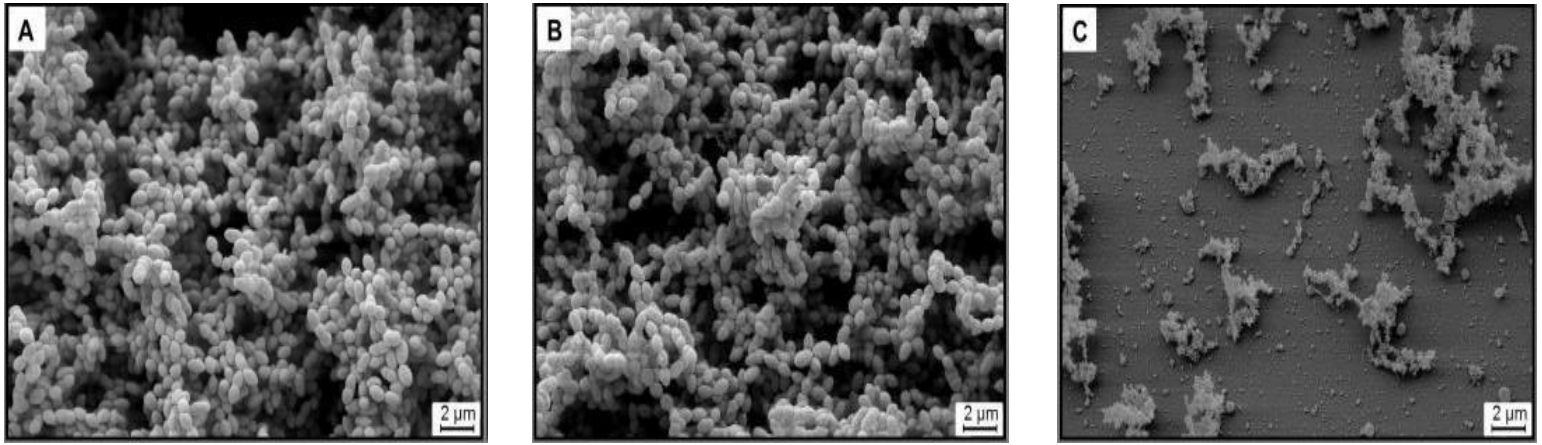
Pneumococcal biofilm formation with…

no inhibitor | **Oseltamivir, c = 10 µM** | **cpd 27, c = 10 µM**



confirmation of biofilm inhibition by compound 27

Grienke U, Richter M, Walther E, Hoffmann A, Kirchmair J, Makarov V, Nietzsche S, Schmidtke M, Rollinger JM. Sci Rep 2016; 6, 27156

Electron microscopy studies

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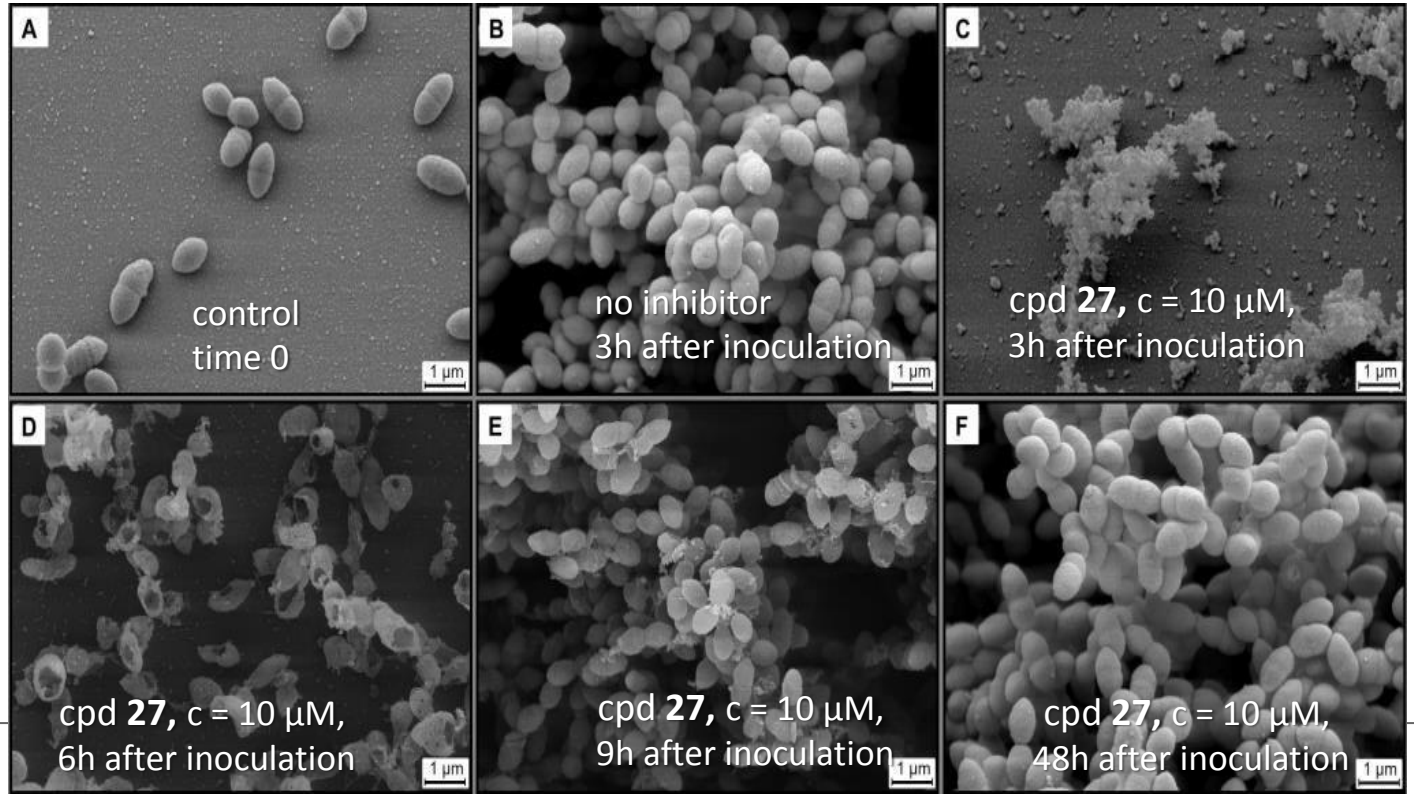
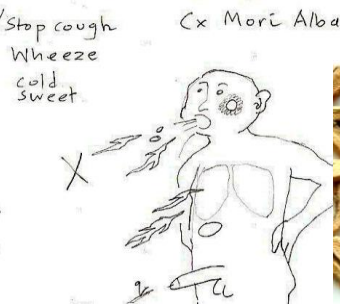
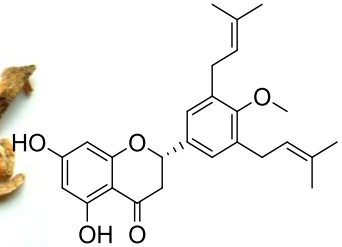
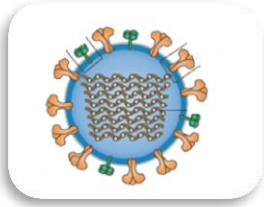
Time dependent inhibition of pneumococcal biofilm

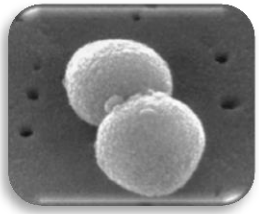


Killing two birds with one stone!









- Prenylated NP & derivatives (**5**, **27**) reduce viral yield in A549 cells in the presence & absence of bacterial NA
- → new lead structures to combat the lethal synergism between influenza viruses and pneumococci
- Mulberry root bark constituents rationalize long-standing application in TCM

Grienke U, Richter M, Walther E, Hoffmann A, Kirchmair J, Makarov V, Nietzsche S, Schmidtke M, Rollinger JM. Sci Rep 2016; 6, 27156

Thanks for your attention!

Department of Pharmacognosy

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Universitätsklinikum Jena

Natural lead structures targeting influenza

FWF Der Wissenschaftsfonds.

FluProtect

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