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Human Hyal-1 – from *in silico* pharmacophore modeling to *in vitro* inhibitor screening

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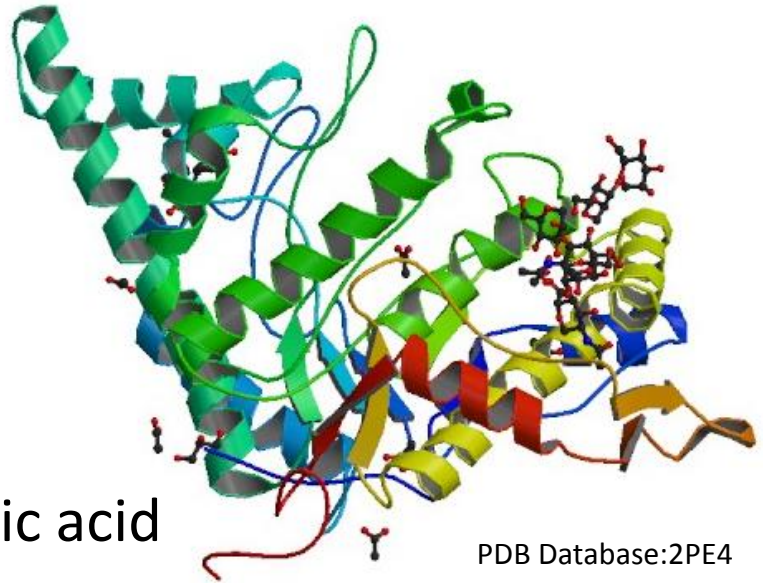
Abstract: The endoglycosidase hydrolase Hyaluronidase 1 (Hyal-1) is one of five hyaluronidases in human body. Degradation of high molecular weight hyaluronan (HA) is mainly catalyzed by Hyal-1 into smaller fragments. These fragments have inflammatory and angiogenic effects.[1] The role of Hyal-1 in cancer progression, e. g prostate or bladder cancer, has been discussed for a long time. In several cancer types, the expression level of Hyal-1 was elevated in comparison to not malignant cells, resulting in higher Hyal-1 activity and tumor progression.[2,3] Although Hyal-1 is an interesting target for pharmaceutical purposes, no potent inhibitors have been found so far. The enzyme source seems to be the bottleneck in investigation of potent inhibitors. Production of active Hyal-1 is one of the most challenging tasks. Eukaryotic extraction and purification is very time consuming and expensive. Recombinant expression in bacteria leads to inactive Hyal-1 forming inclusion bodies. Therefore, potent Hyal-1 inhibitors, like chemical compounds or plant extracts, are routinely screened against bovine testis hyaluronidase, which has an amino acid sequence identity of approx. 40 % compared to human Hyal-1. This again causes problems in interpretation of the obtained data, development of a pharmacophore model or searching for leader compounds inhibiting human Hyal-1. Using Autodisplay technology, we are able to express human Hyal-1 on the surface of *Escherichia coli* in an active form.[4] With this system, it is possible to screen compounds, directly using the desired target. A combination of pharmacophore modeling followed by docking studies using a virtual system and Hyal-1 crystal structure, helped us to get first impressions about binding of the substances to Hyal-1. Next, screening the best hits with whole-cells displaying Hyal-1 seems to be a promising way to find the needle in the haystack.

THE ENZYME - Human Hyaluronidase Hyal-1

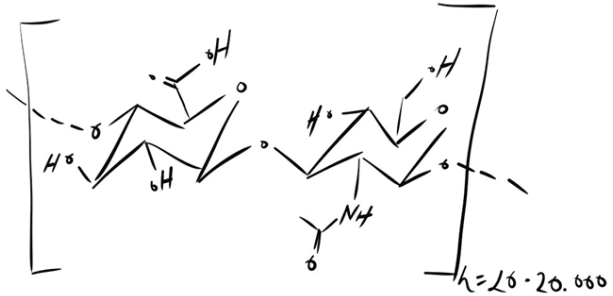


PDB Database:2PE4

- one of five human hyaluronidases
- 4-glycosaminohydrolase
- Catalyzing hydrolysation of hyaluronic acid
- Lysosomal enzyme
- Increased expression of *hyal-1* mRNA and higher amounts of Hyal-1 are documented in bladder and prostate cancer

THE SUBSTRATE – Hyaluronic acid

A polysaccharide with diverse physiological functions



glucuronic acid N-acetylglucosamine

no degradation

Hyal-1

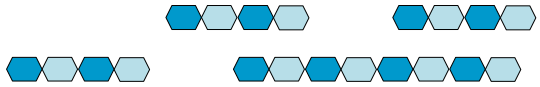
- antiproliferativ
- immunosuppressiv
- antiangiogenic
- space filling

High molecular weight HA (HMW HA >20 kDa)

degradation

Hyal-1

- inflammatory effects
- angiogenic



Low molecular weight HA (LMW HA <20 kDa)

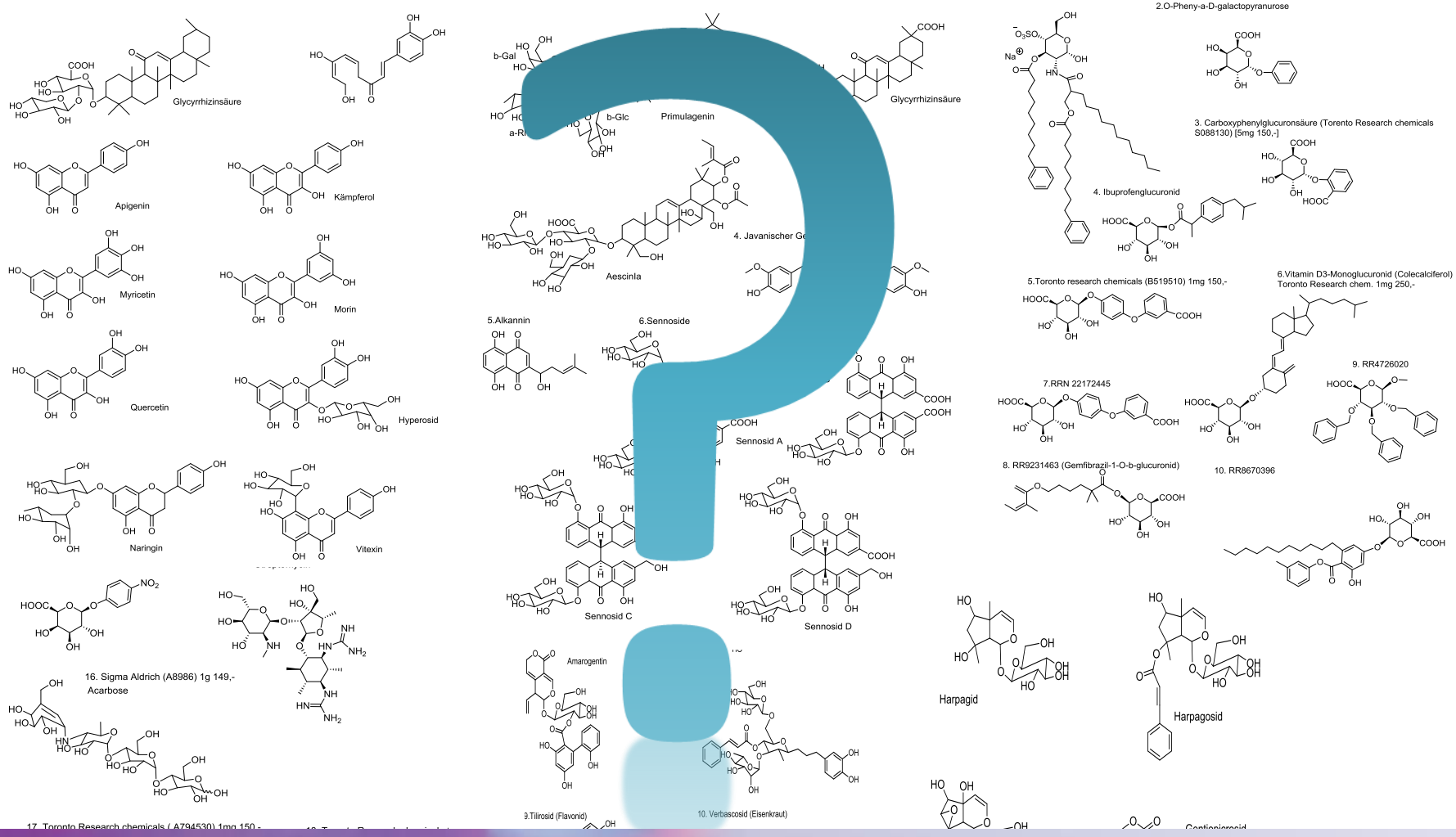
PHARMACEUTICAL APPLICATIONS OF HIGH MOLECULAR WEIGTH HYALURONIC ACID

- Nose spray for moistening the nose mucosa
- Tablets or pastilles against sore throat
- Eye drops against dry eyes
- Gels and mouth waters against gingivitis
- Joint injections against rheumatoid arthritis
- Subcutaneous injections against winkles
- Countless care cosmetics, against wrinkles or dry skin

THE HYPOTHESIS – Inhibition of Hyal-1

- prevents cleavage of high molecular weight HA into smaller fragments with pathophysiological effects, like
    - Angiogenic and inflammatory effects
- may also help to get a step further to the youth of the skin

THE TASK – Identification of compounds inhibiting Hyal-1



THE BOTTLENECK – ENZYME SOURCE

Problem:

- Production and purification out of *Drosophila Schneider-2* cells (*DS*2-cells):

  → **low yield, high costs and time consuming**

- Production and purification out of *E. coli* cells:

  → **missing enzyme activity (formation of inclusion bodies)**

THE SOLUTION – AUTODISPLAY

- Autodisplay**:**
  → Expression of Hyal-1 on the surface of *Escherichia coli* cells

THE MECHANISM - AUTODISPLAY

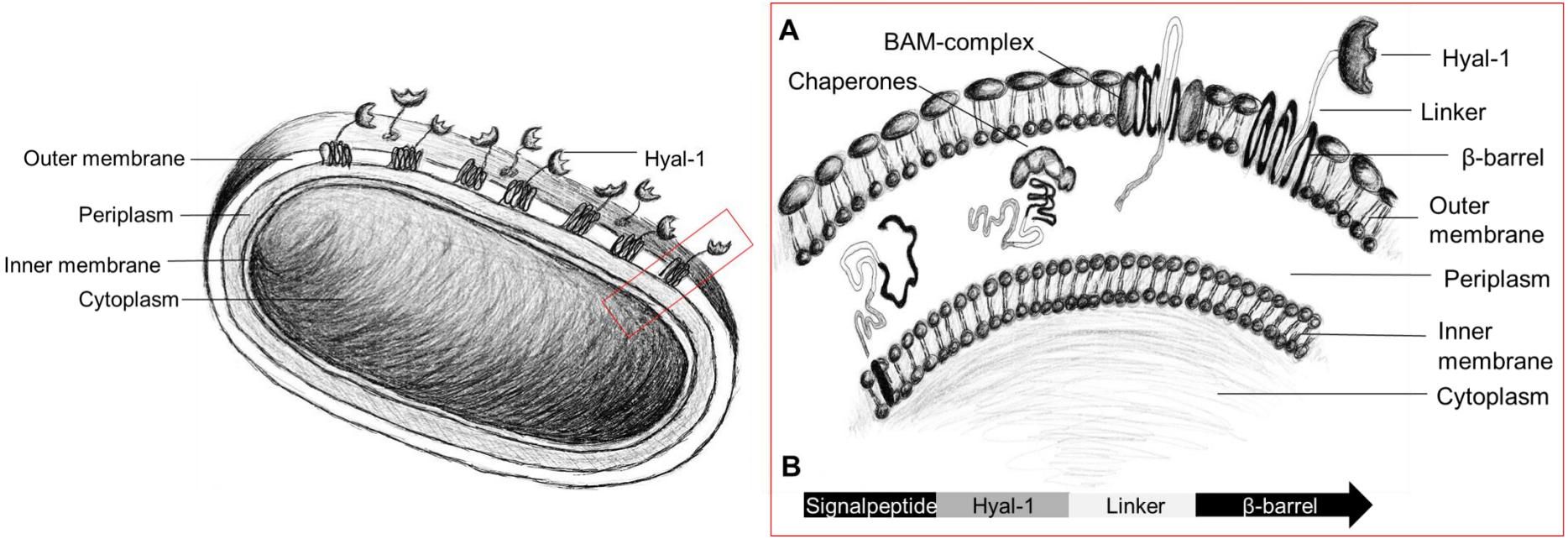
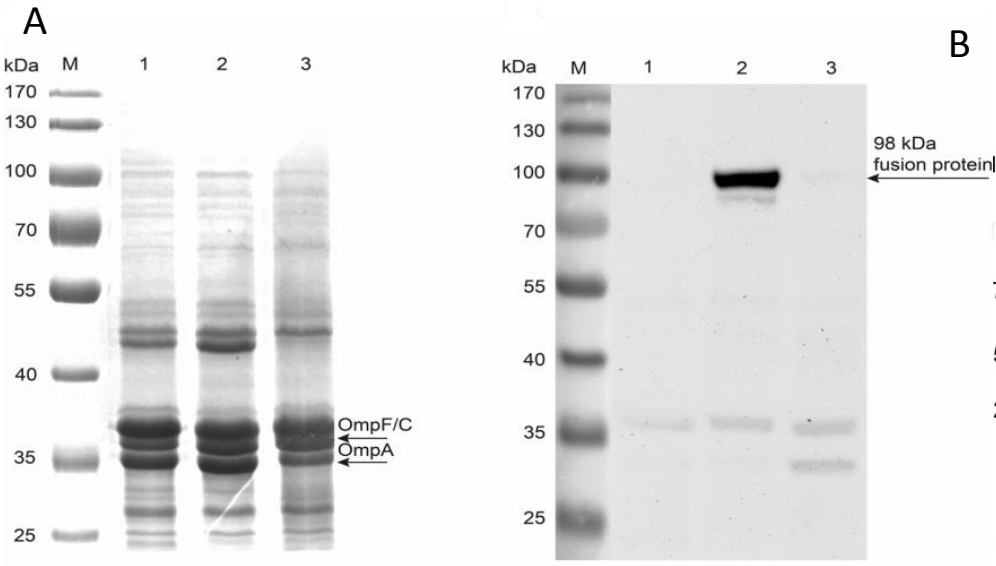
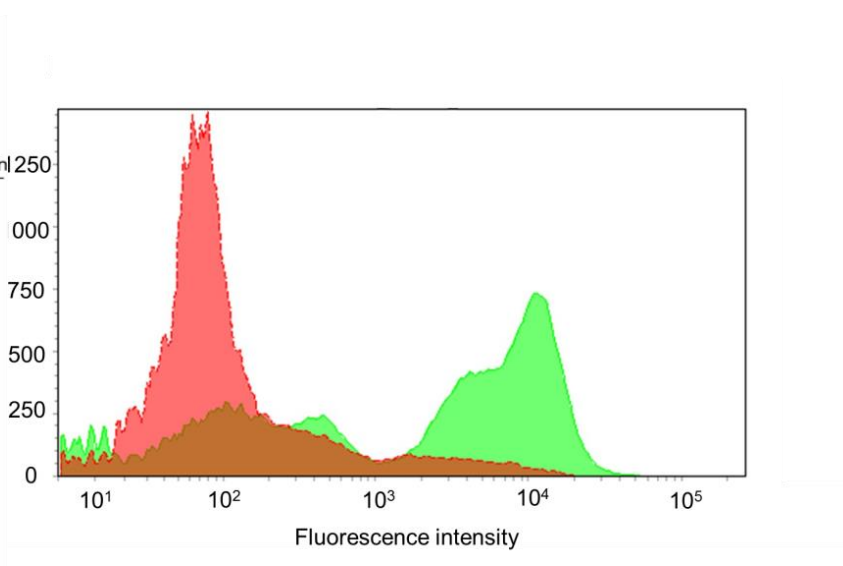


Image of *E. coli* cells displaying Hyal-1 on the surface. In the red square the mechanism of the Hyal-1 fusionprotein translocation is sketched. The Autotransporterfusionprotein is expressed in the cytoplasm, then translocated into the periplasm by a CtxB-signalpeptide. This peptide is then cleaved by a signalpeptidase. The β-barrel is folding into the outermembrane and linker and Hyal-1 are translocated through this membrane. Outside the cell, but still immobilized on the cellmembrane, the Hyal-1 starts to fold properly.

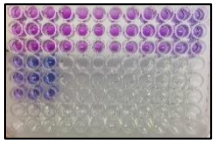
Proof of surface expression of Hyal-1



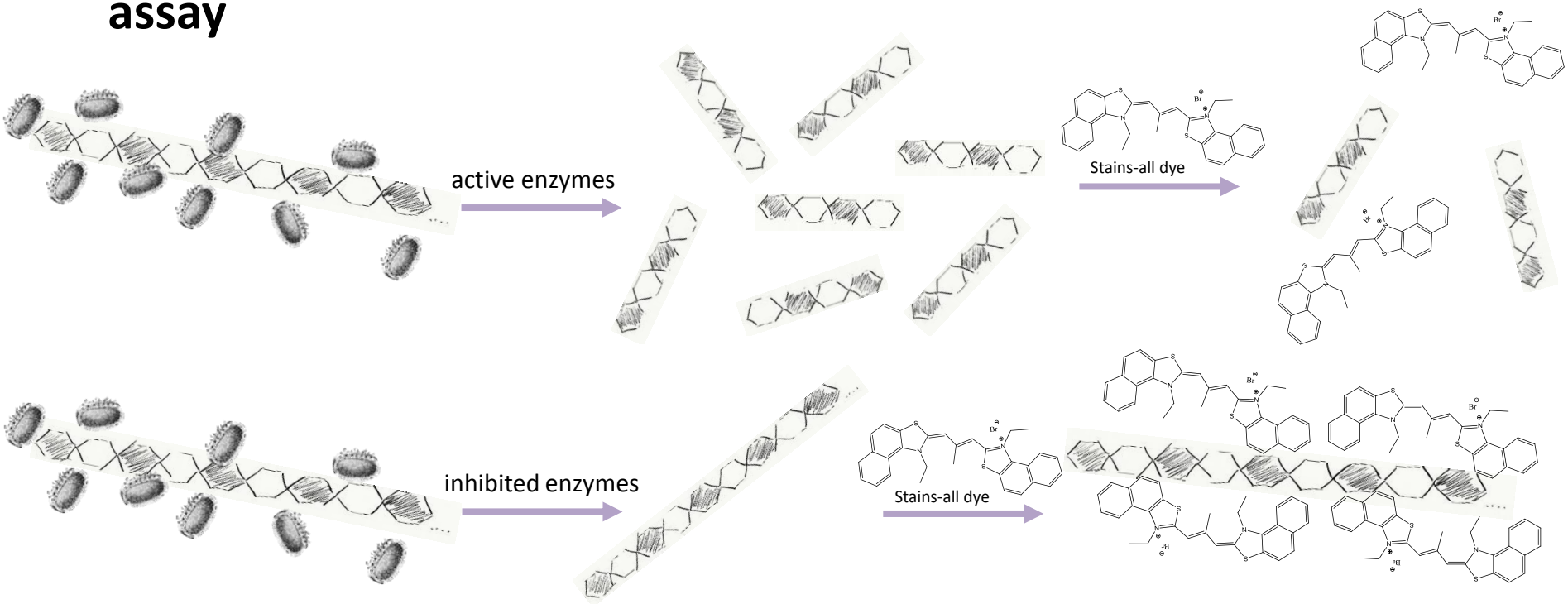


A: Outermembrane proteins were separated *via* SDS-polyacrylamidgel electrophoresesis. The expected band at 100 kDa for Hyal-1 linked to the autotransporter is not obvious in line 2. The following immoblot using a primary anti-hyal-1 antibody determined the Hyal-1 fusionprotein, which indicates a low amount of the enzyme, but it is on the surface of *E. coli*. This could be confirmed by proteinase K digestion in lane 3.

B: Flowcytometric analysis of *E. coli* cells displaying Hyal-1 (green) and *E. coli* cells without Hyal-1 on the surface (red). Both strains were treated with fluoresceinmaleimid, a dye kuppling to cysteins. Hyal-1 has 10 cysteine residues. This is the reason for the increase of fluorescence in figure B.

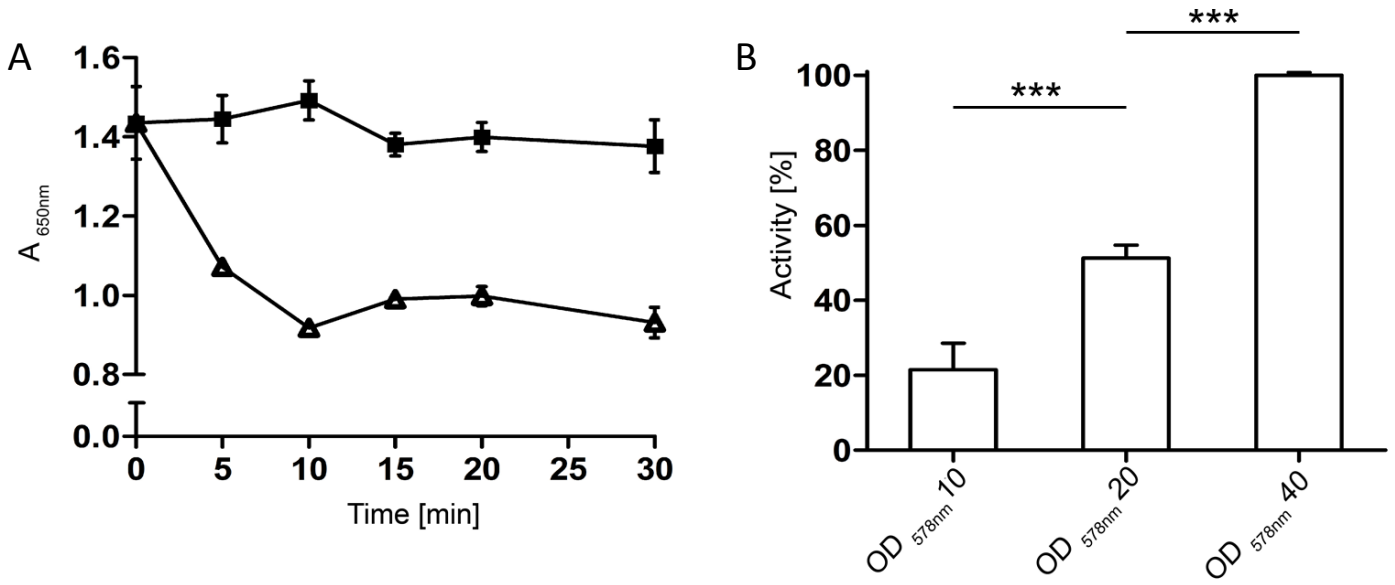


STAINS-ALL ASSY – The photometric whole cell enzyme activity assay



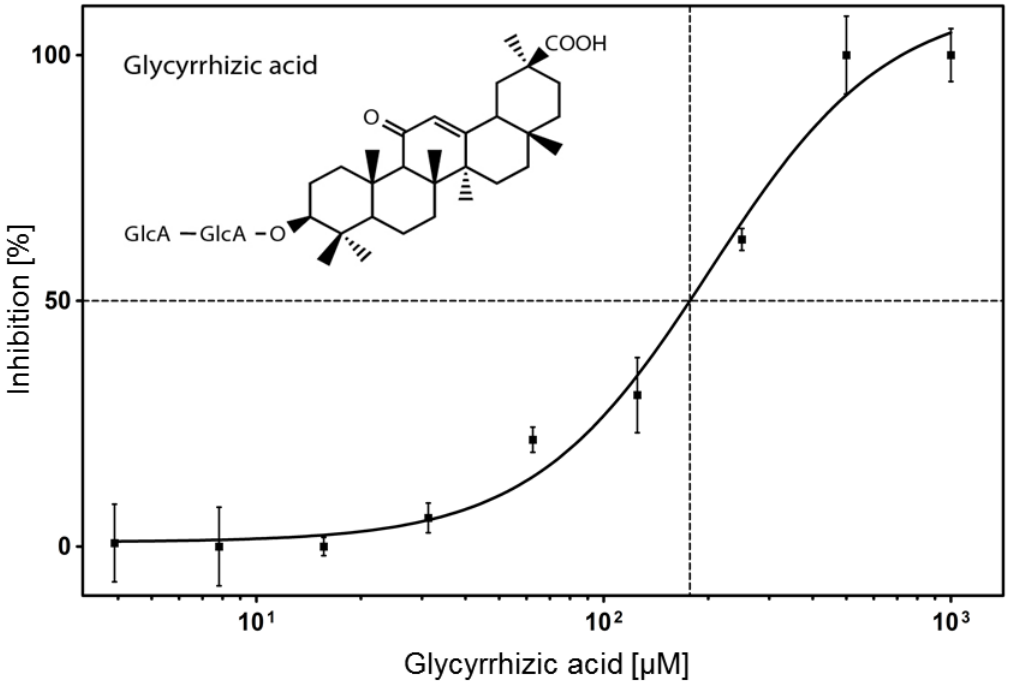
Enzyme activity can be measured by residual high molecular weight Hyaluronic acid amount.The performance, the supernatant of the reactionsolution is treated with Stains-all dye. This dye can only complex high molecular weight hyaluronic acid. This complex shows an absorbance at 650nm.If the high molecular weigth hyaluronic acid is degraded, no complex can be built and no absorbance can be measured, which indicates activity of the immobilized enzyme on the surface of *E. coli.*

Hyal-1 activity: HA degradation depending on time and Hyal-1 amount



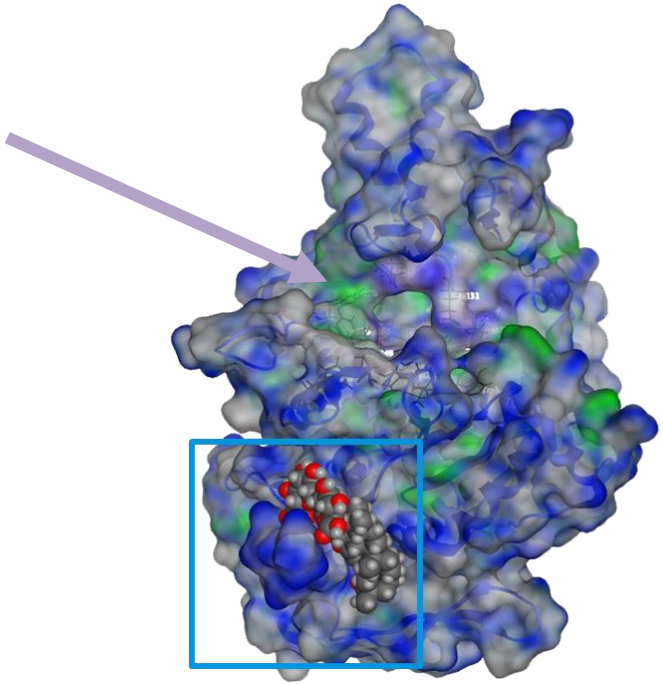
A: Within the first 10 minutes of reaction, a linear decrease of absorbance could be observed for cells displaying Hyal-1 (triangle), the control cells without the enzyme on the surface show no effect. For inhibitor screening the time point within this linear sector is necessary and was chosen to be 5 minutes. B: Increasing the amount of cells (optical densitiy, OD_{578nm}), means increasing amount of enzyme which leads to an increased activity.

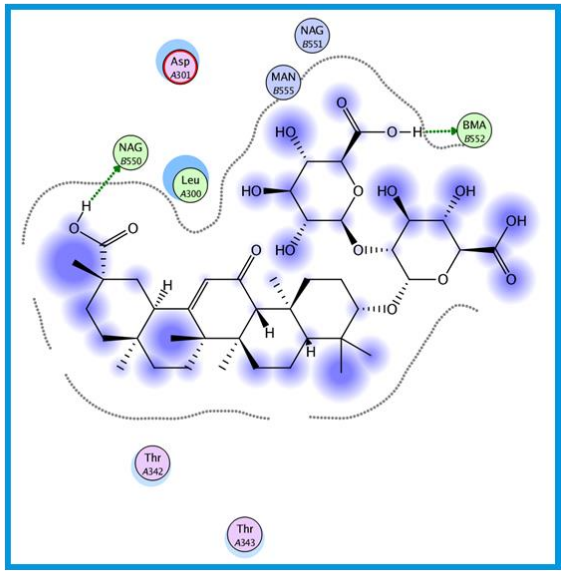
Glycyrrhizic acid as Hyal-1 reference inhibitor



Glycyrrhizic acid as reference inhibitor used to validate the assay. An IC_{50} value of 171µM was determined with good accordance to literature values.

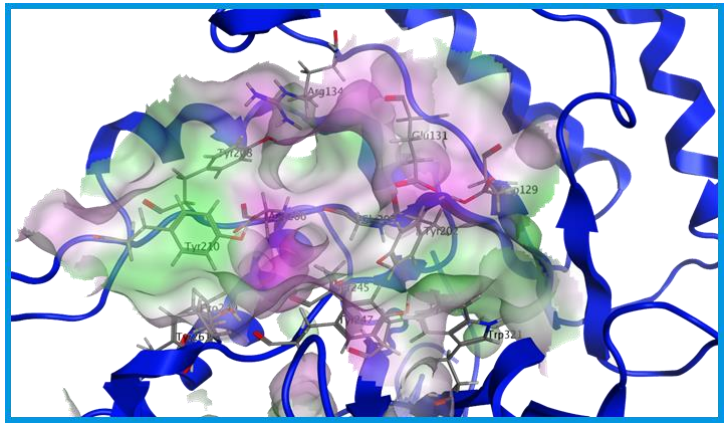
In silico docking studies on Hyal-1





Docking studies on Hyal-1 (cristallstructure 2PE4) using glycyrrhizic acid as ligand showed binding of the glycyrrhizic acid. Having a look at the binding position the ligand binds not at the active site of the enzyme (purple arrow) bit at a allosteric site. Looking closer to the ligand-enzyme-interactions (blue square), the ligand seems to interact with the glycosylated sugar moieties. This has to be further investigated, because the recombinant expressed Hyal-1 in bacteria does not have any glycosylation sites, but never the less, glycyrrhizic acid, shows an allosteric inhibition mode.

In silico pharmacophore modeling on Hyal-1 for identification of new ligands



The pharmacophore model was based on the aminoacids in site the active site of Hyal-1. Several amino acids were shown to have an influence on the activity of the enzyme. These amino acids were used to generate a pharmacophore model with 7 features. After, the ZINC® database was screened and the pharmacophore was used as a filter. About 200 compounds could be detected and after conformational search docked into Hyal-1. The best fits now should be tested in the in vitro assay to confirm the results of the in silico experiments and also to have new lead structures for Hyal-1 inhibitors.

CONCLUSION

Hyal-1 is a very interesting target, but there are a lot of barriers to overcome searching for a potent inhibitor.

A combination of *in silico* and *in vitro* experiments seems to be the best opportunity for enzymes, which are not well known.

The data determined by computational measurements can give the right hints for identification of compounds inhibiting Hyal-1, but have to be confirmed by *in vitro* experiments.

Many thanks to

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