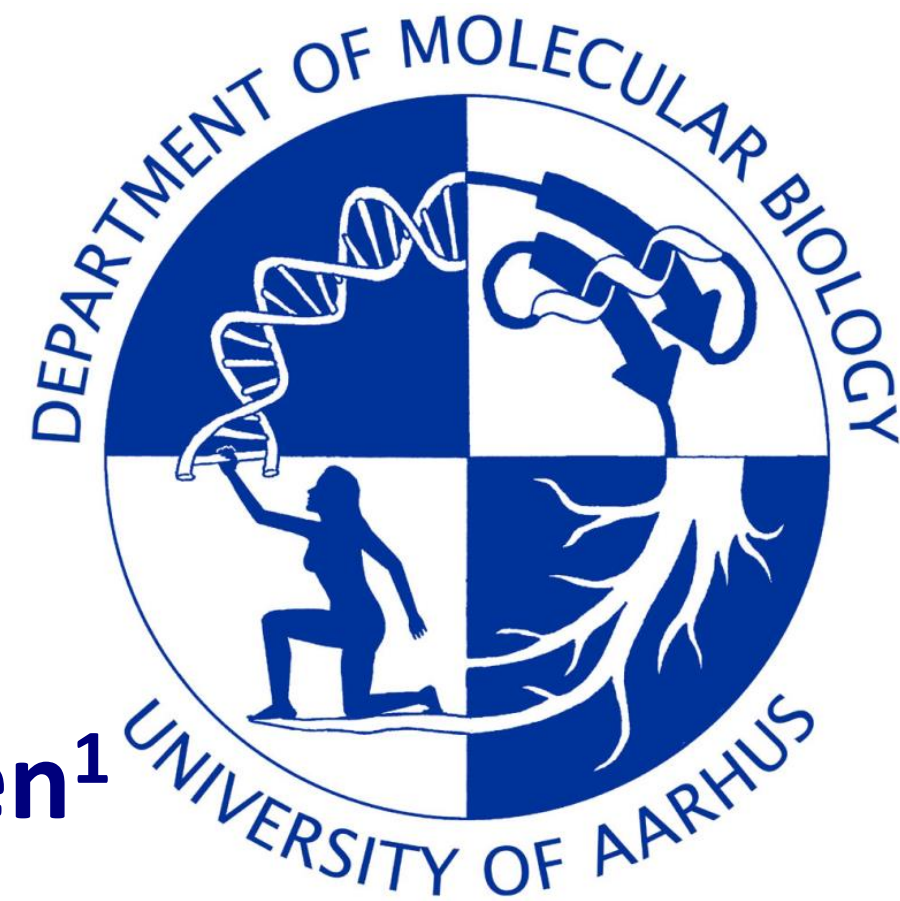




New potential therapeutic applications of WX-UK1, as a specific and potent inhibitor of human trypsin-2 and human trypsin-3

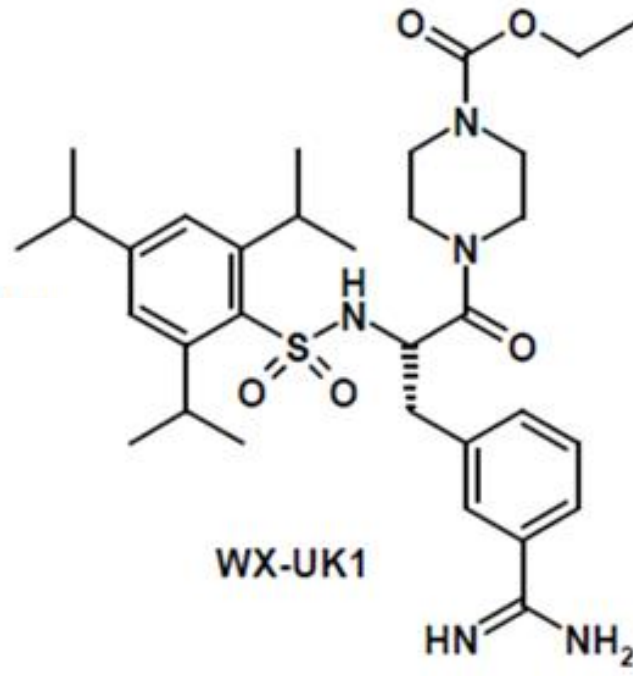


Emil Oldenburg¹, Christine R. Schar¹, Eva L. Lange¹, Danielle Abramson², Terry F. Plasse², Eric M. Towler², Reza Fathi², Mark Levitt^{2*}, and Jan K Jensen¹

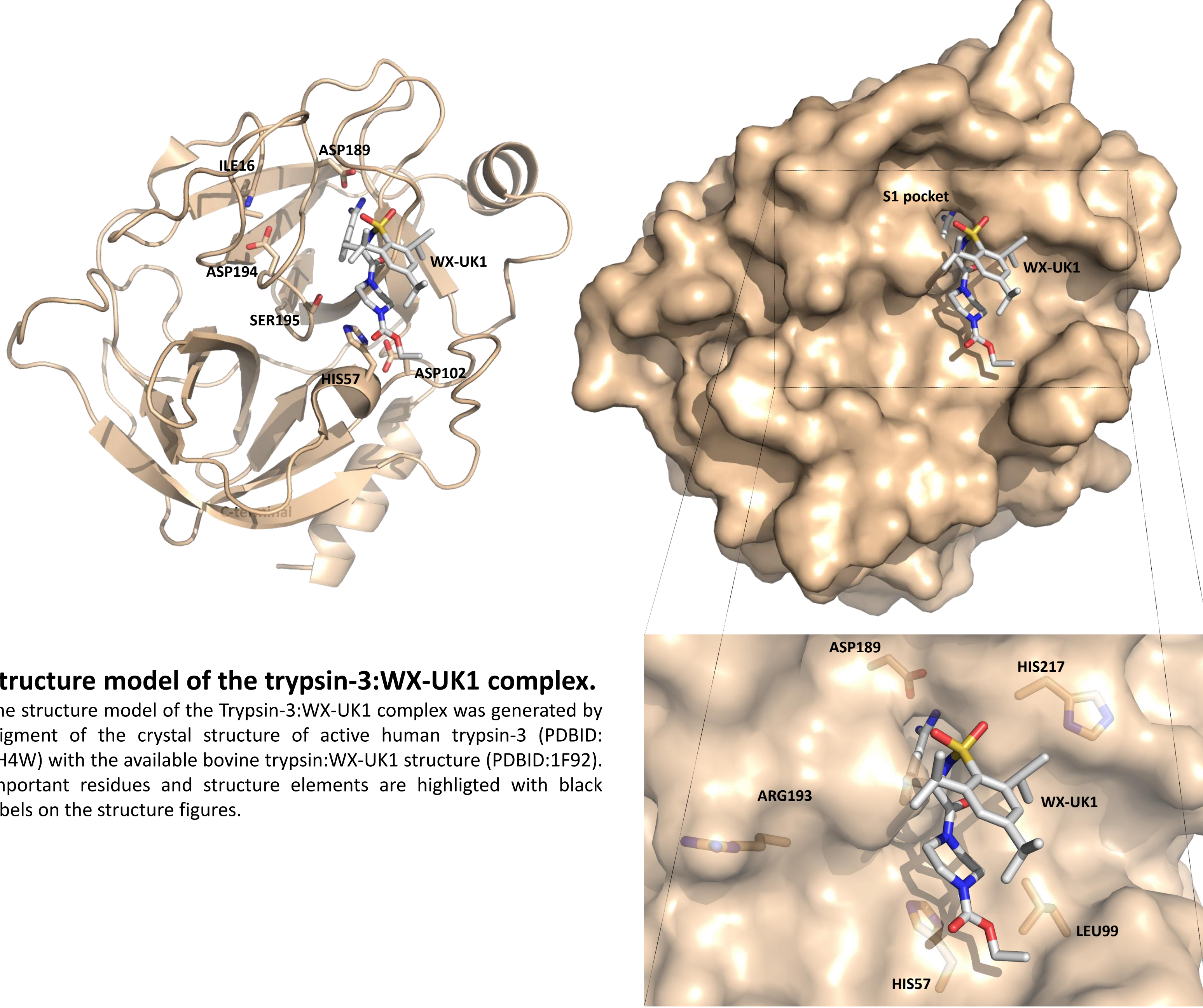
¹Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds vej 10, 8000 Aarhus, Denmark. ²RedHill Biopharma, Ltd. 21 Ha'arba'a St. Tel Aviv 6473921, Israel.

BACKGROUND

Until recently, WX-UK1 was known primarily as a synthetic small molecule inhibitor of the serine protease urokinase (uPA). We now reveal that WX-UK1 is a potent and rather specific inhibitor of human trypsin-2 and human trypsin-3, and propose that these new molecular targets represent new therapeutic applications, in addition to its cancer therapeutic potential.



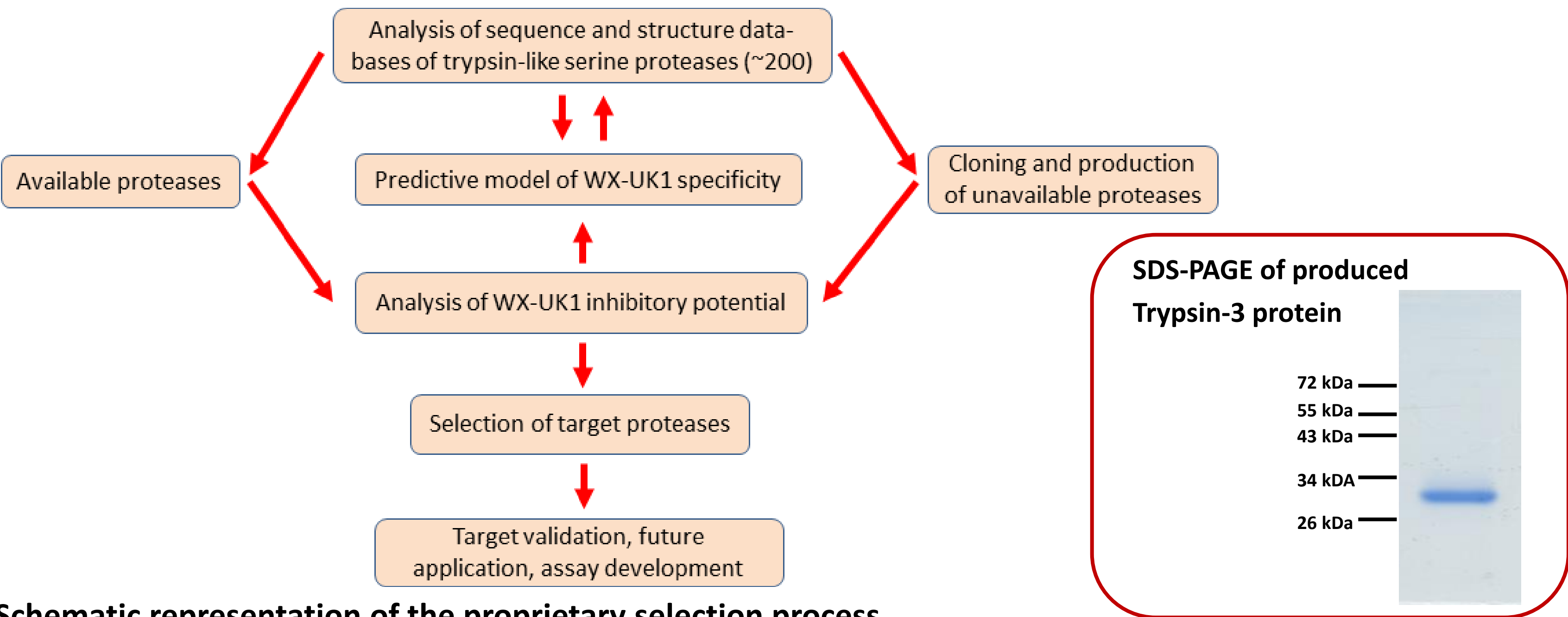
WX-UK1 binds directly to the active site of chymotrypsin-like proteases with trypsin specificity (ARG/LYS), acting as a competitive inhibitor



Structure model of the trypsin-3:WX-UK1 complex.

The structure model of the Trypsin-3:WX-UK1 complex was generated by alignment of the crystal structure of active human trypsin-3 (PDBID: 1H4W) with the available bovine trypsin:WX-UK1 structure (PDBID:1F92). Important residues and structure elements are highlighted with black labels on the structure figures.

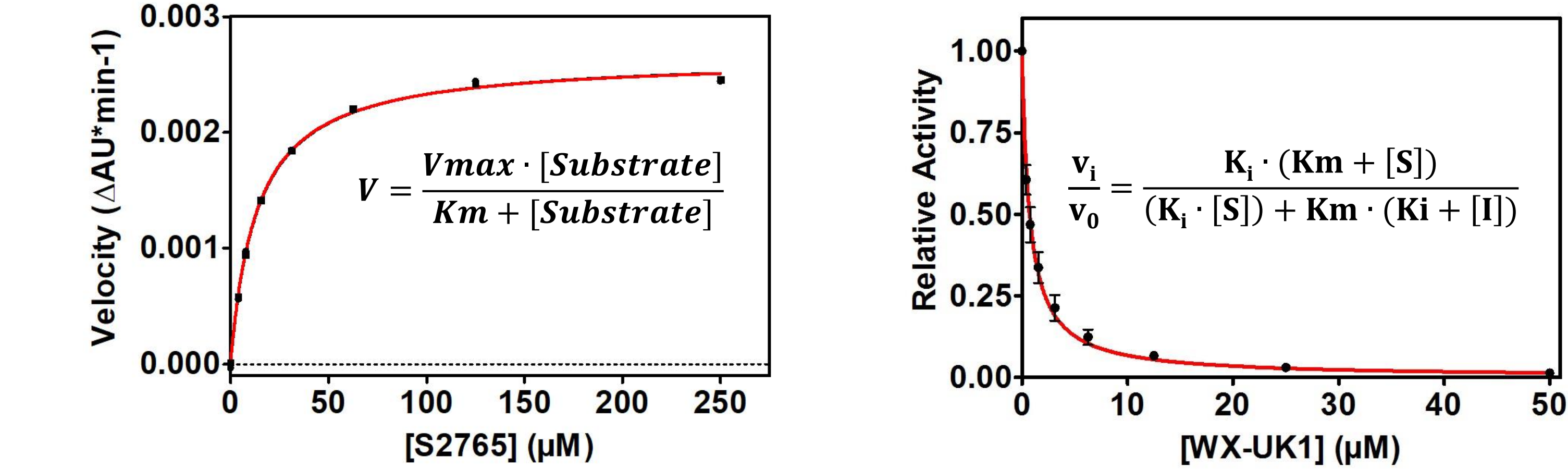
Selection of promising WX-UK1 targets among the chymotrypsin-like serine proteases in the human genome



Schematic representation of the proprietary selection process.

Trypsin- 2 and Trypsin-3 were selected as targets for inhibition by WX-UK1 among the ~200 serine proteases in the Human genome using a proprietary approach. The starting point of the approach was a handful of proteases for which WX-UK1 was described to inhibit with varying potency. based on bioinformatic and biochemical characterization. By a thorough analysis of the sequence database and available structural information of all human chymotrypsin-like serine proteases with trypsin-like specificity, a circular iterative process was carried out in which structure and sequence based criteria for WX-UK1 inhibition was tested by measuring WX-UK1 inhibition of selected protease. Based on the biochemical data, the database was repetitively reanalysed to improve the selection criteria (insert 1). The end result was that fewer than ten human serine proteases were identified as promising WX-UK1 targets for further characterization with respect to possible treatment of pathological conditions. Proteases not commercially available were produced from bacteria, yeast or higher eukaryotic cells using in-house procedures (insert 2). The identity, purity and activity of all proteases, including purchased, were verified by Mass spectroscopy and biochemical analysis

WX-UK1 inhibition of selected serine proteases was characterized by K_i determination



Selected proteases were produced, a suitable chromogenic substrate was chosen and the inhibitory constant for inhibition by WX-UK1 was determined using an enzymatic assay.

All chromogenic enzyme assays were performed at 37°C in 30mM HEPES pH 7.4, 150mM NaCl supplemented with 0.5 (W/V) % BSA. The chromogenic substrate with the lowest K_m was chosen (left graph) and used for the determination of the inhibitory constant for WX-UK1 (K_i). In the K_i experiment, the enzyme activity is measured in the presence of a concentration series of WX-UK1 (right graph). A fixed substrate concentration equal to or higher than the K_m was used in the K_i experiment to ensure enough competition with WX-UK1 to allow for an accurate K_i to be determined using the competitor equation (right insert). The K_i values of relevant proteases are given in table 1 (above).

Protease	K _i (μM)
Human uPA	0.9 ± 0.1 (3)
Human Trypsin-1	0.19 ± 0.01 (3)
Human Trypsin-2	0.075 ± 0.003 (6)
Human Trypsin-3	0.019 ± 0.004 (6)

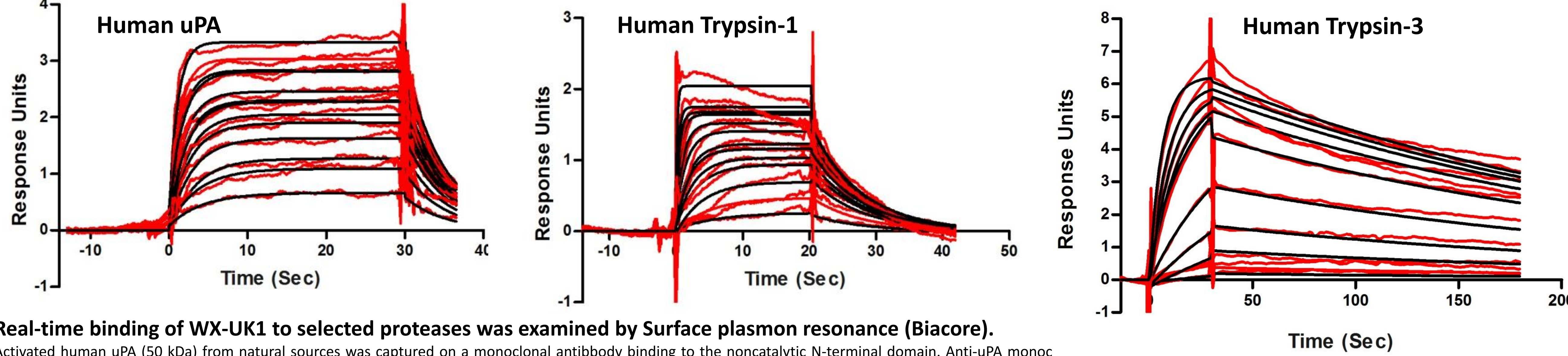
Table 1. Table of relevant inhibitory constants (K_i) determined during this investigation. The K_i values are the mean of more than 3 independent determinations (n) and a standard deviation (SD).

PERSPECTIVES

WX-UK1 was originally developed as an inhibitor of uPA and other members of the S1 family of trypsin-like serine proteases, essential to aspects of tissue remodeling associated with tumor invasion and metastasis. With the identification of human trypsin-3 and human trypsin-2 as high affinity molecular targets, new disease indications may be proposed in addition to cancer.

As a low nanomolar inhibitor of human trypsin-3, WX-UK1 may find potential utility in the treatment of inflammatory digestive diseases, including irritable bowel syndrome, inflammatory bowel disease and pancreatitis, the latter for which there are currently no approved therapies. Furthermore, WX-UK1, as a human trypsin-2 inhibitor could potentially be used in the treatment of inflammatory lung diseases, including acute respiratory distress syndrome, acute lung injury, α-1 antitrypsin deficiency and chronic obstructive pulmonary disease (COPD), the latter of which represents a major unmet medical need.

The drop in K_i from Trypsin-1 to Trypsin-3 is associated with a reduced dissociation rate in support of a tighter protease interaction



Real-time binding of WX-UK1 to selected proteases was examined by Surface plasmon resonance (Biacore).

Activated human uPA (50 kDa) from natural sources was captured on a monoclonal antibody binding to the noncatalytic N-terminal domain. Anti-uPA monoc Trypsin-3 (27 kDa) was captured on a CM5 chip using the standard amine-coupling protocol. Pushing the limit of sensitivity of the Biacore T200 by studying the binding of the <700 Dalton WX-UK1, the theoretical number of protease active sites in each experiment was between 5 - 0.5 fmol/mm² to support measurable binding. All binding experiments were performed at 25°C, at a flow rate of 30μl/min in 30mM HEPES pH 7.4, 150mM NaCl supplemented with 0.5 (W/V) BSA and 0.05% (V/V) Tween20. The resulting data from global fit of a 1:1 binding model are given in table 2 for relevant tested proteases.

Human trypsin-(1-3) share high sequence homology, but distinct variation at key positions predicted to govern WX-UK-1 binding specificity

	16	20	30	40	50	60	70	80	90	99	100	110	120	130	140	150	160	170	180	189	193	200	210	217	220	230	240	250	
Trypsin-1:	IVGGY	NCEENSVPYQ	VSL-----	NSGYH	FCGGSLINEQ	VVVSAGHCYK	---SR---	IQVRLGE	HNIEVLEGNE	QFINAAKIIR	HPQYDRK	--TLN	NDIMLIKLS	----	RAVINARVST	ISLPT-APPA	-TGTKCLISG	WGNTASSGAD	YPDELQCLDA	PVLSQAKCEA	--SYPGKITSNM	FCVGFLEGGKDS	CQGDSSGGPVV	CNQG----	LQ	GVVSWGDCGA	QKNRPGVYTK	VYNYVKWIKN	TIAANS----
Trypsin-2:	IVGGY	ICEENSVPYQ	VSL-----	NSGYH	FCGGSLISEQ	VVVSAGHCYK	---SR---	IQVRLGE	HNIEVLEGNE	QFINAAKIIR	HPKYNRS	--TLD	NDILLIKLS	----	PAVINSRVS	ISLPT-APPA	-AGTESLISG	WGNTLSSGAD	YPDELQCLDA	PVLSQAQCEA	--SYPGKITNNM	FCVGFLEGGKDS	CQGDSSGGPVV	SNGE----	LQ	GIVSWGYCA	QKNRPGVYTK	VYNYVDWIKD	TIAANS----
Trypsin-3:	IVGGY	TCEENSLPYQ	VSL-----	NSGSH	FCGGSLISEQ	VVVSAAHCYK	--TR---	IQVRLGE	HNIEVLEGNE	QFINAAKIIR	HPKYNRD	--TLD	NDIMLIKLS	----	PAVINARVST	ISLPT-TPPA	-AGTECLISG	WGNTLSFGAD	YPDELQCLDA	PVLTQAQCKA	--SYPGKITSNM	FCVGFLEGGKDS	CQGDSSGGPVV	CNQG----	LQ	GVVSWGCGA	WKNRPGVYTK	VYNYVDWIKD	TIAANS----
uPA	:	IIGGE	FTTIENQPWF	AAIYRRHRGGSVTY	VCGGSLISPC	WVISATHCFI	DYPKEDYIVYLR	SRLNSNTQGE	MKFEVENLIL	HKDYSADTLAHH	NDIALLKIRS	KEGRCAQPSRTIQT	ICLPSMYNDP	QFGTSCITEIG	FGKENSTDYL	YPEQLKMTVV	KLISHRECCQ	PHYYGSEVTTKM	LCAADPQWKTD	S	CQGDSSGGPLV	CSLQGRMTLT	GIVSWGRCGA	LKDKPGVYTR	VSHFLPWIRS	HTKEENGLAL			
																										</			

Sequence alignment of human trypsins and human uPA.

The presented sequences cover the catalytic domain starting from Ile16 (chymotrypsin template numbering), the first residue after the activation site. Key residues for prediction of WX-UK-1 binding and residue 193 responsible for the inhibitor resistance of trypsin-3 are marked. Sequence homology to trypsin-1 is shown the right panel.