2022 rbc REGIONAL BIOPHYSICS CONFERENCE

Dipeptidyl Peptidase III pathophysiological role(s) in human

Involvement of Dipeptidyl Peptidase III in Oxidative Stress and Pain Regulation

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hrzz Hrvatska zaklada za znanost

- **Family of DPP IIIs** (M49, **zinc** dependent metalo enzymes widely spread).

- **5 kingdoms** Eubacteria, Protist

HEXXGH

EEXR(K)AE(D)

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SP|Q9NY33|DPP3_HUMAN   TQRE--KLTFL-EEDDKDLYILWKGPSFDVQVGLHELL-GHGSGKLFVQDEKGAFNFDQE 474
SP|Q08225|DPP3_YEAST   SSKH--PPSFI-SQEDRPIFEKYQSDSFEVQVGIHELL-GHGSGKLLTEF-TDGFNFDKE 483
TR|Q8A6N1|Q8A6N1_BACTN AHGNGFNEEFVCNDEERQRIDQYGDLTGELHTDLHECL-GHGSGKLLPGVDPD------- 465
TR|Q7MX92|Q7MX92_PORGI ARGTGLYEEFIPDEEVRRHVELHADLTDSLHTDLHECL-GHGSGQLLPGVPGD------- 449
TR|H1XW48|H1XW48_9BACT LLKP--IAEKVLFAEQLPLVT---FEGFFNHTLMHEISHGLGPGKIVLNG---------- 394
TR|A9TLP4|A9TLP4_PHYPA ILLP--IANVCVEASQRGAVD---FDSFFTHTICHECCHGIGPHNIVTPD--G------- 607
                                      .             :.  **   * *   :

SP|Q9NY33|DPP3_HUMAN   TVINPETGEQIQSWYRSGETWDSKFSTIASSYEECRAESVGLYLCLHPQVLEIFGFEGAD 534
SP|Q08225|DPP3_YEAST   NPPLGLDGKPVSTYYKVGETWGSKFGQLAGPFEECRAEVIAMFLLTNKKILDIFGFHDVE 543
TR|Q8A6N1|Q8A6N1_BACTN ----------------------ALKAYGSTIEEARADLFGLYYVADPKLVELKLVPDAE 502
TR|Q7MX92|Q7MX92_PORGI ----------------------ALGEHASTLEETRADLFALYFLADPKMIELGLLTDPD 486
TR|H1XW48|H1XW48_9BACT ----------------RQTEVKKELKETYSSIEECKADVLGMYNNL--FMIEKGVYP--P 434
TR|A9TLP4|A9TLP4_PHYPA ----------------RASTVRLELQEVYSAIEEAKADIVGLWALH--FLVDKGLLP--R 647
                                        :    .  ** :*: ..::      ::
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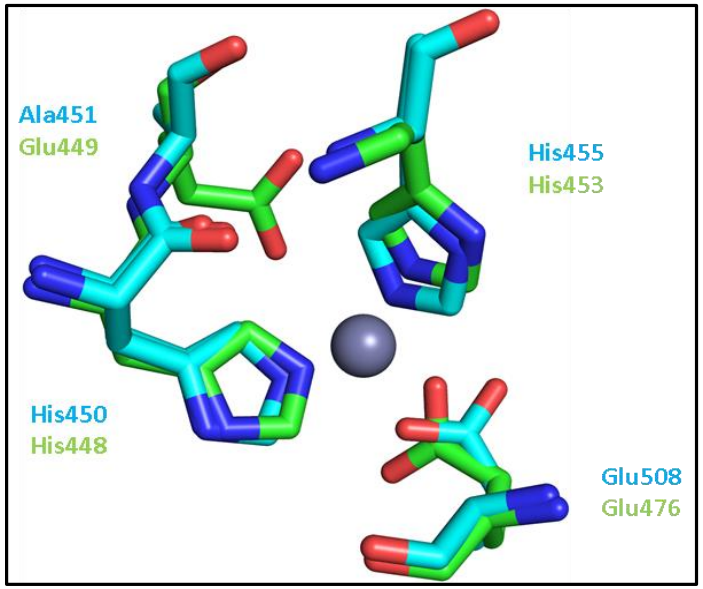
DPP III orthologs

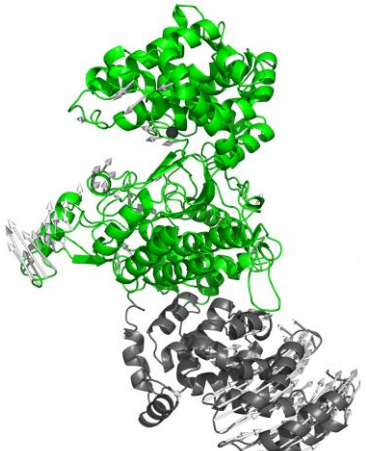
Percent of amino acid identity matrix

	hDPPIII	yDPPIII	BtDPPIII	PgDPPIII	PpDPPIII	CaDPPIII
hDPPIII	100	36.4	23.95	23.65	19.52	22.72
yDPPIII	36.4	100	21.77	22.69	17.41	19.33
BtDPPIII	23.95	21.77	100	49.92	22.89	22.31
PgDPPIII	23.65	22.69	49.92	100	20.34	22.09
PpDPPIII	19.52	17.41	22.89	20.34	100	42.21
CaDPPIII	22.72	19.33	22.31	22.09	42.21	100



2008. → **yeast** DPP III (PBD id 2CSK)
2009. → **human** DPP III (PBD id 3FVY)

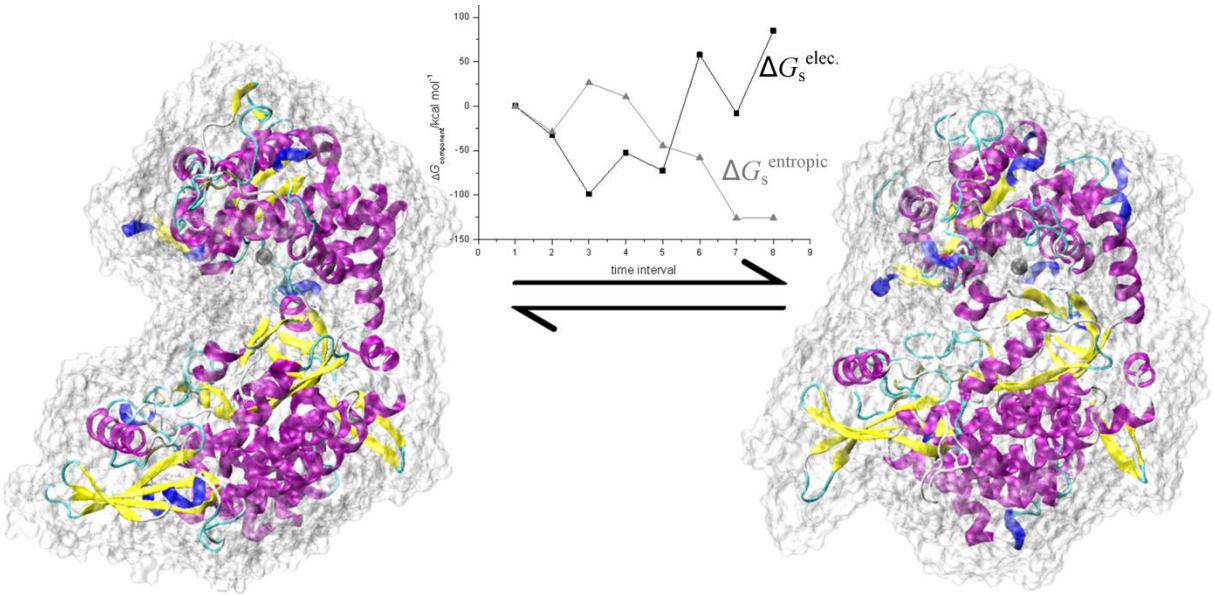
Molecular mass ~61-103 kDa

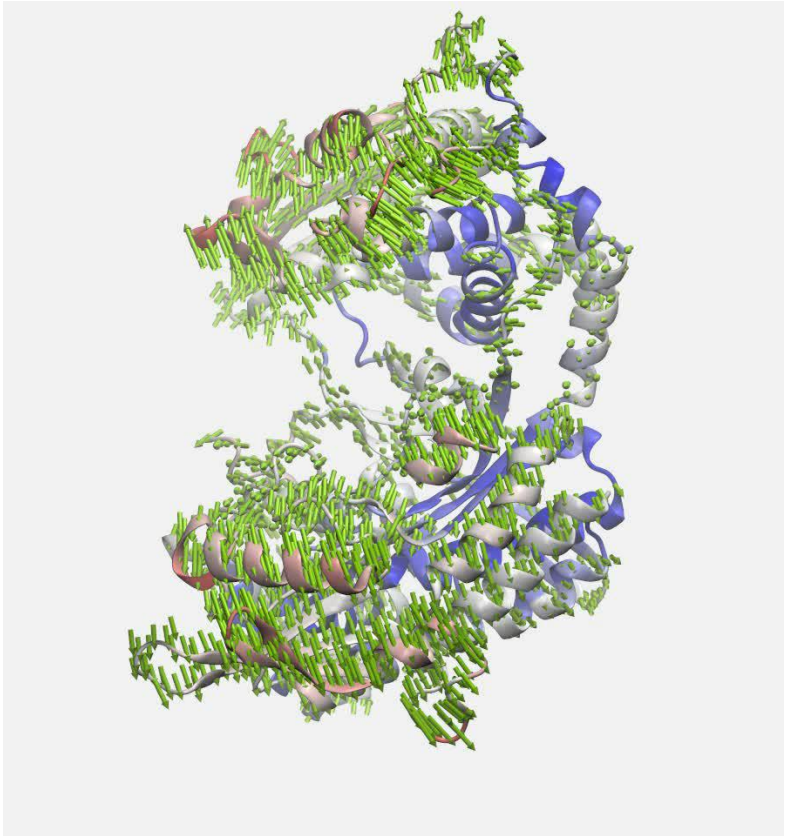


Zn^{2+} crucial for activity coordinated by AA from motifs **H**EXXG**H** and E**E**XR(K)AE(D)

RSC Adv. **8** (2018), 13310-13322.

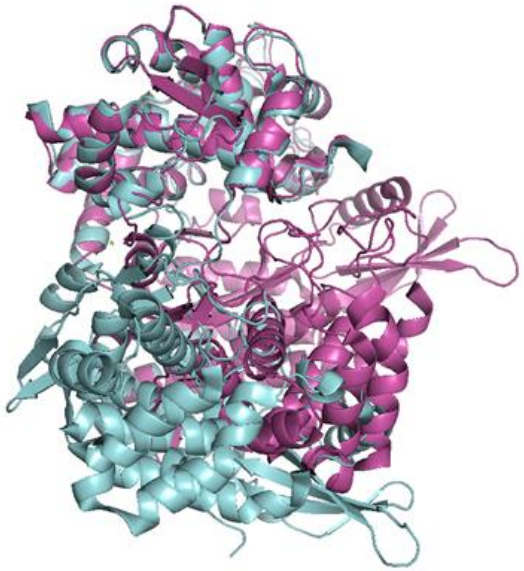
Long range motion of human DPP III





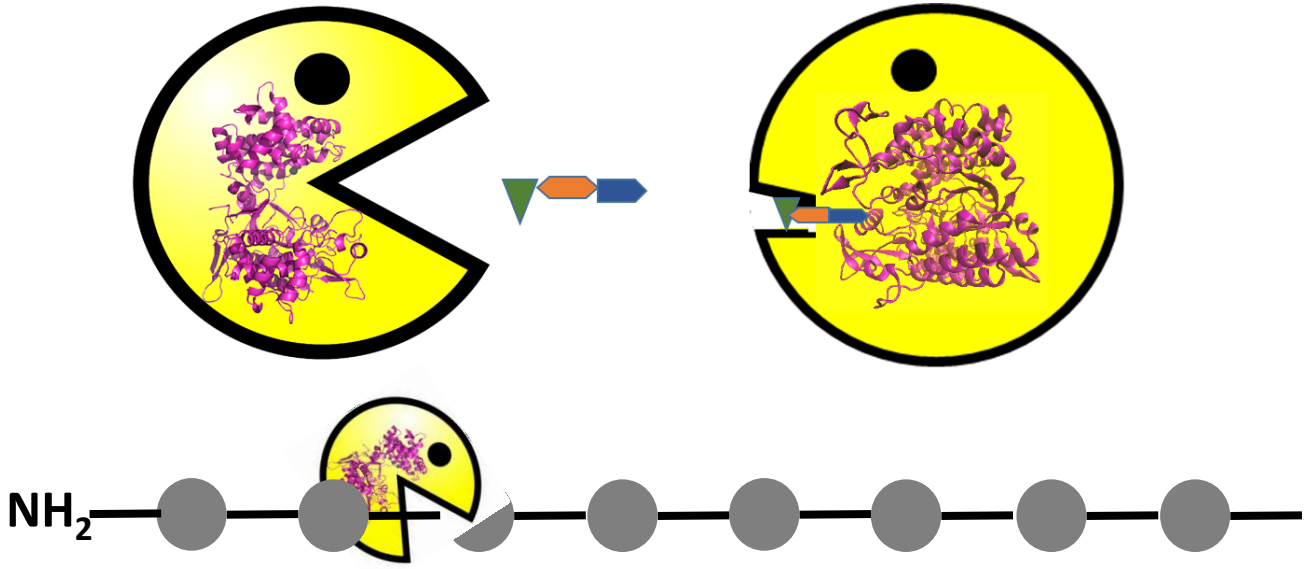
Mol. BioSyst. **11** (2015); 3068-3080

human DPP III (PBD id 3FVY)
E451A human DPP III with tynorphin (PBD id 3T6B)
human DPP III (PBD id 5EGY)



Human Dipeptidyl Peptidase III

PEPTIDASE ACTIVITY OF DPP III
Involvement in the pain regulation



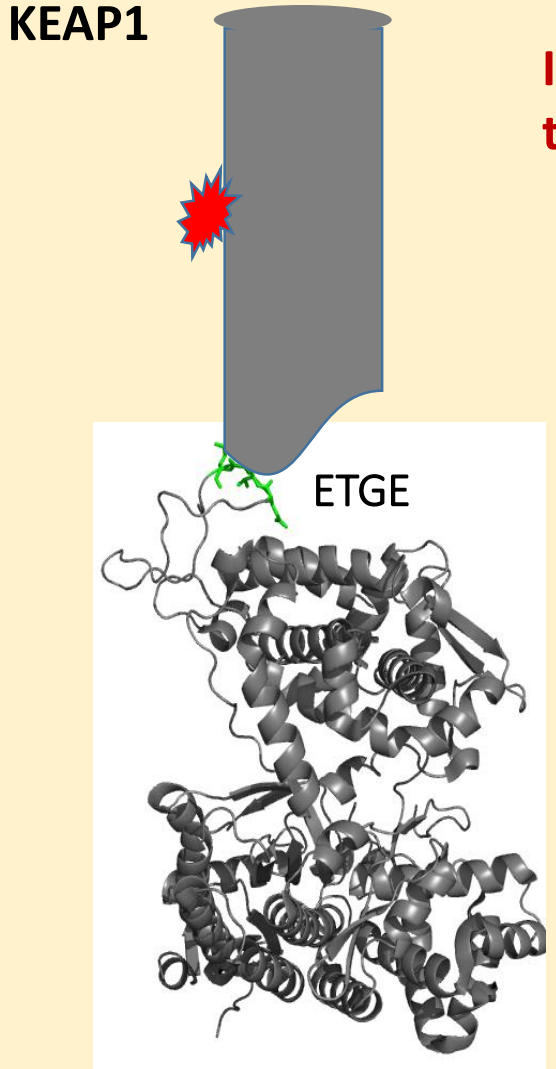
Angiotensin II → **Blood pressure**

Cruz-Diaz et al. *Peptides.* **83** (2016) 29–37.
Pang et al. *Hypertension*. **68** (2016) 630–641

Neuropeptides → **PAIN**

Baršun et al. *Biol. Chem.* **388** (2007) 343–348;
A. Tomić, B. Kovačević, S. Tomić, *Phys. Chem. Chem. Phys*. **18** (2016) 27245–27256

Protein-protein interacton



Involvement in the oxidative stress

Hast et al. *(Cancer Res.* ***2013****;73(7), 2199)* determined DPP III as an interactor of KEAP1, the main sensor of the oxydative stress in cell

Dipeptidyl Peptidase III in neurophatic disorders

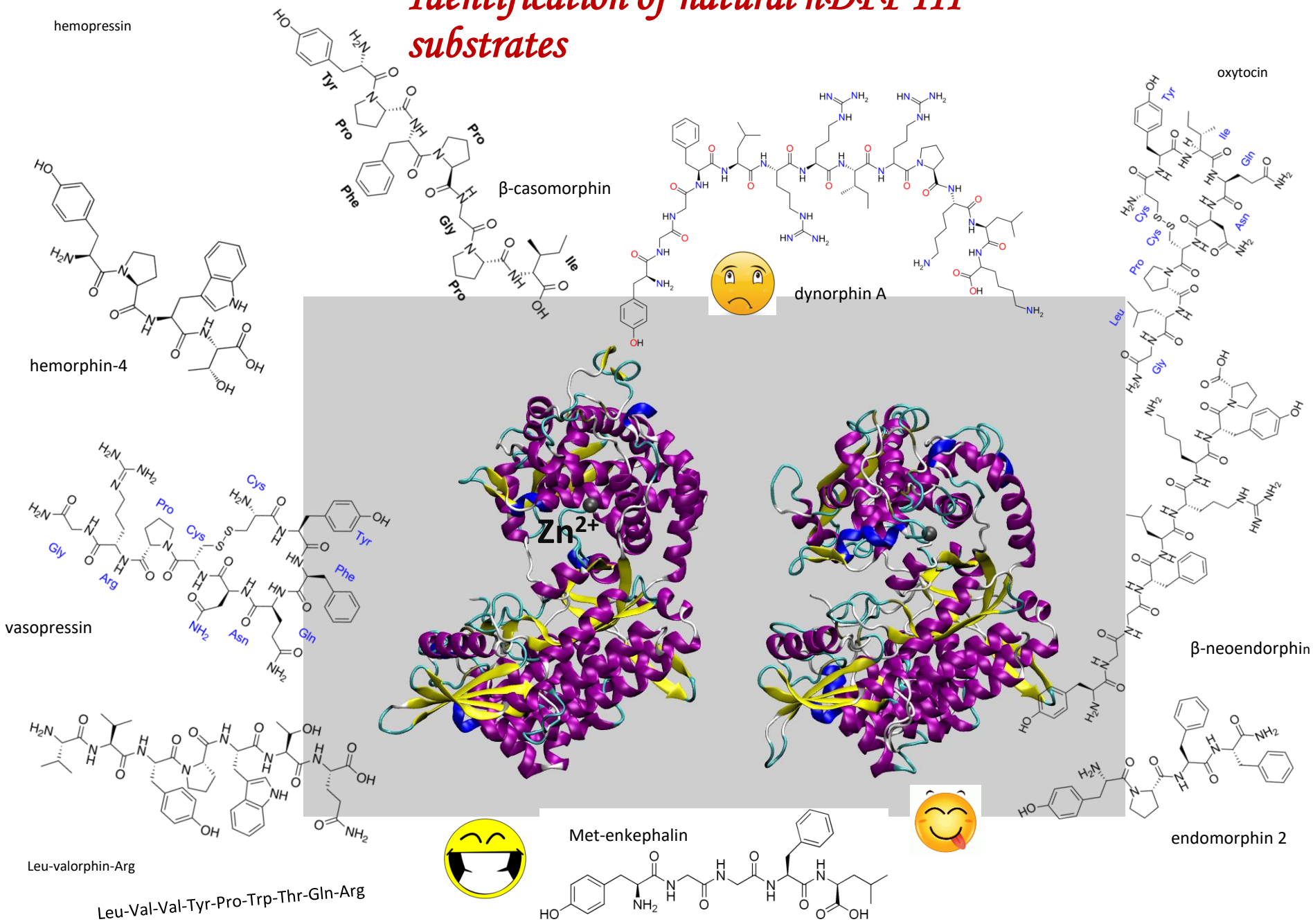
The high concentration of DPP III in the rat spinal cord, where enkephalin-synthesizing neurons are located, suggests its role in the mammalian pain regulatory system (T. Chiba, et al. *Peptides.* **24** (2003) 773–778).

Sato *et al*. (*Japanese J. Anesthesiol*. **52** (2003) 257–263) found that the activity of DPP III in cerebrospinal fluid of patients with pain differs from that without pain.

In 2008 Thanawala *et al*. (*Curr. Drug Targets.* **9** (2008) 887–894) suggested DPP III as a potential target for pharmacological treatment of pain, and
in 2018, Buckley *et al*. (*Mol. Neurobiol.* **55** (2018) 2420–2430) found that the DPP3 gene is regulated differently in patients with CNP than in the normal population.

In addition, the endogenous heptapeptide spinorphin and its truncated form tynorphin have been found to inhibit DPP III peptidase activity, and Ueda *et al*. (*Peptides.* **21** (2000) 1215–1221) demonstrated that spinorphin can induce analgesia in mice.

Identification of natural hDPP III substrates



Neuropeptides substrates (inhibitors) of DPP III

HPLC-MS, ITC and fluorimetric measurements

peptide	sequence	HPLC-MS (cleaved)[a]	K_d / µM[b]	K_i / µM[c]
I-tynorphin	IVYPW	Y	0.0973 ± 0.0091	0.00045 ± 0.00005
S-tynorphin	SVYPW	Y	0.298 ± 0.061	0.0077 ± 0.0007
tynorphin	VVYPW	Y	0.386 ± 0.127	0.0112 ± 0.0008
valorphin	VVYPWTQ	Y	1.78 ± 0.21	0.0365 ± 0.0029
angiotensin II	DRVYIHPF	Y	2.22 ± 0.24	4.4 ± 0.5
Leu-valorphin-Arg	LVVYPWTQR	Y	2.50 ± 1.92	5.2 ± 0.5
hemorphin-4	**YPWT**	**Y**	**39.4 ± 14.6**	**6.5 ± 0.7**
endomorphin-2	YPFF	Y	40.1 ± 4.8	10.4 ± 1.0
Leu-enkephalin	YGGFL	Y	118 ± 39	10.4 ± 1.4
β-casomorphin	YPFVEPI	Y	130 ± 87	1.0 ± 0.1
Arg-vasopressin	C*YFQNC*PRG	N	n. d.	n. d.[d]
hemopressin	PVNFKFLSH	N	n. d.	n. d.
β-neoendorphin	YGGFLRKYP	N	n. d.	n. d.
dynorphin A (1-8)	YGGFLRRI	50% ?	n. d.	n. d.

* - denoting a disulfide bridge

[a] – cleavage (Y yes or N no) determined by HPLC-MS as reduction of peptide amount after incubation of 1 mM peptide with 0.18 µM enzyme after 24 h at 25 °C in ammonium bicarbonate buffer pH = 7.4

[b] – Thermodynamic parameters of peptide binding to human DPP III at 25 °C and pH = 7.5 in 20 mM TrisHCl buffer

[c] – inhibition constant for inhibition of enzyme-catalyzed cleavage of artificial substrate Arg_2-2NA at 25 °C in 20 mM TrisHCl buffer pH = 7.5

[d] – no inhibition trend detected with peptide in the range of 1-50 µM

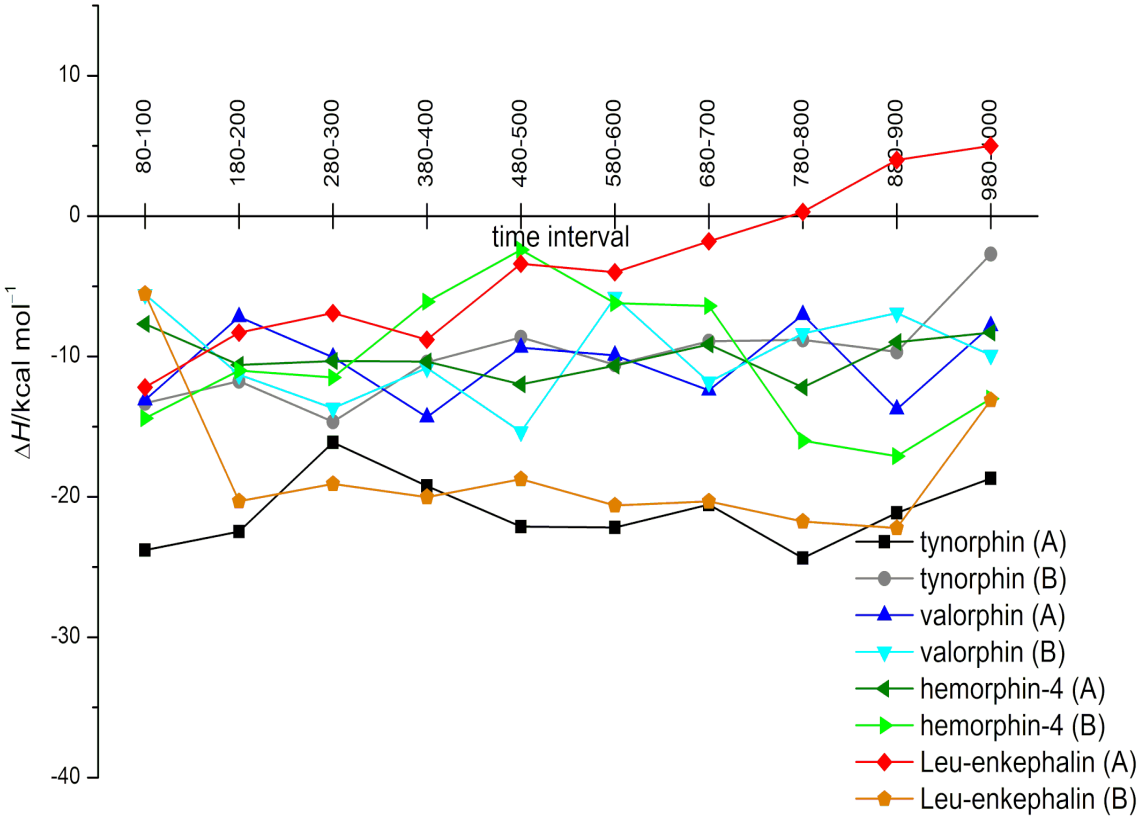
Neuropeptides substrates of Dipeptidyl Peptidase III

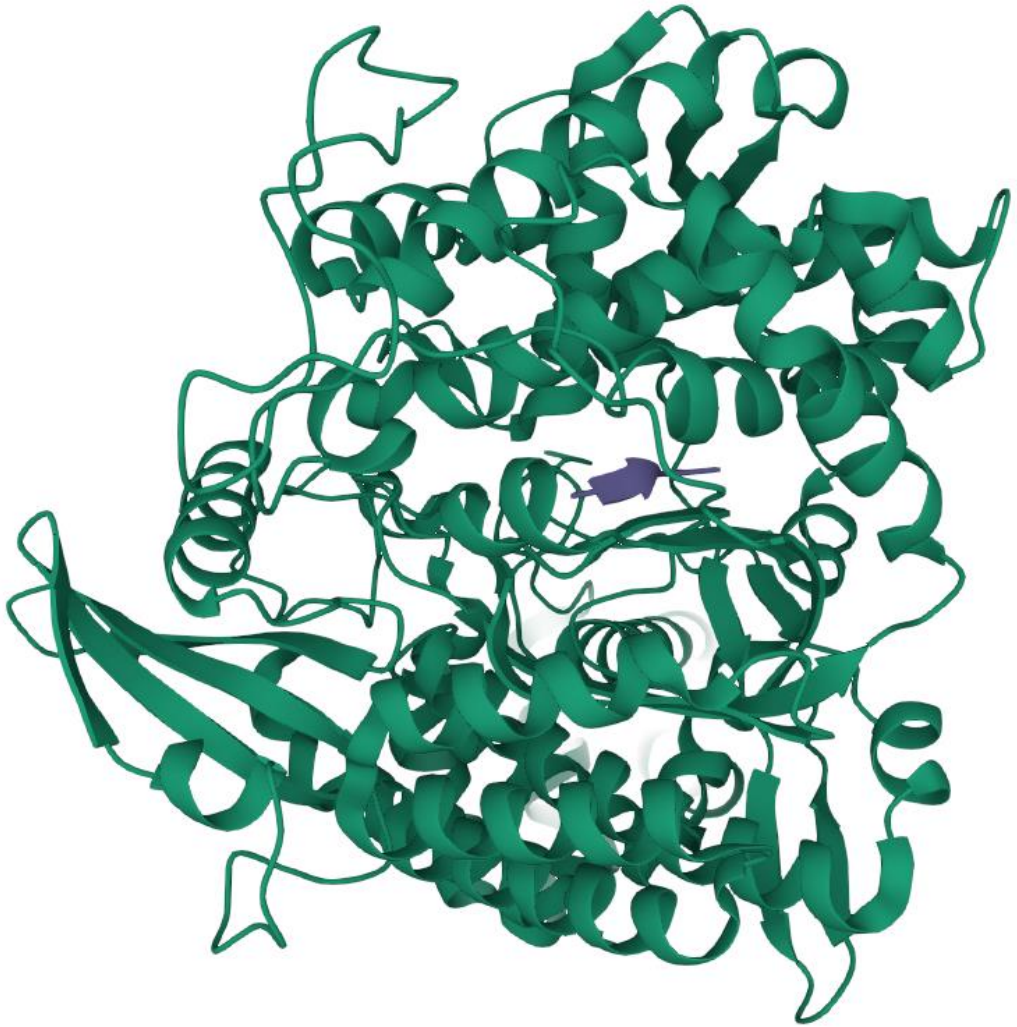
Kinetic parameters of peptide degradation as measured by ITC using SIM at 25°C in 50 mM TrisHCl buffer with 100 mM NaCl and pH 7.5

	$\Delta_r H$ (kJ/mol)	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\ M^{-1}$)
Leu-valorphin-Arg	-6.4 ± 0.3	33.9 ± 6.4	0.35 ± 0.09	$1.03 \cdot 10^4$
Leu-enkephalin	-6.55 ± 0.07	34.7 ± 5.7	1.08 ± 0.12	$3.11 \cdot 10^4$
hemorphin-4	-7.5 ± 0.7	55.1 ± 13.1	6.11 ± 0.96	$1.11 \cdot 10^5$

MD simulations (2x1μs)

MM/PBSA energies calculated during two 1 μs long MD simulations of the DPP III in complexes with selected neuropeptides



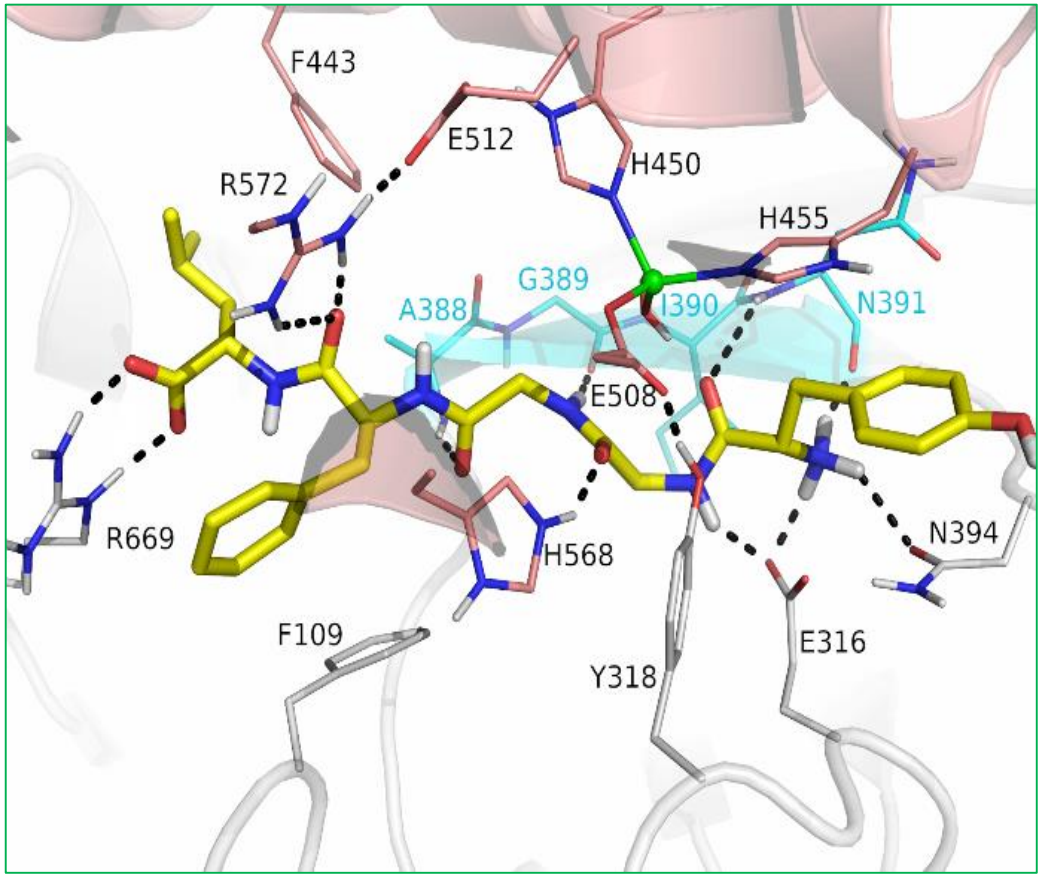


Karačić et al. Neuropeptides, substrates and inhibitors of human dipeptidyl peptides III, experimental and computational study - a new substrate identified, *IJBM*, under review

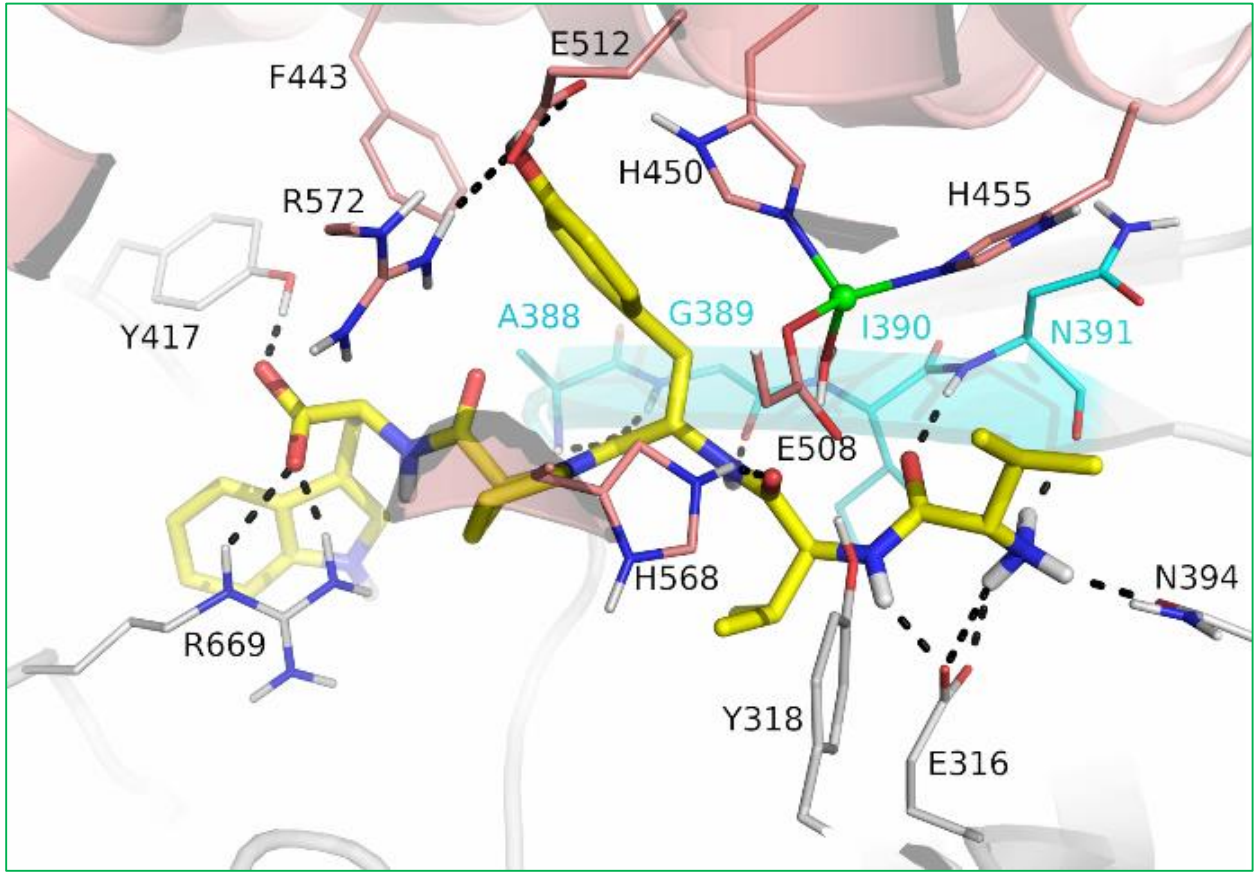
MD simulations

Low energy structure of the

a) DPP III – hemorphin-4 complex



b) DPP III – tynorphin complex



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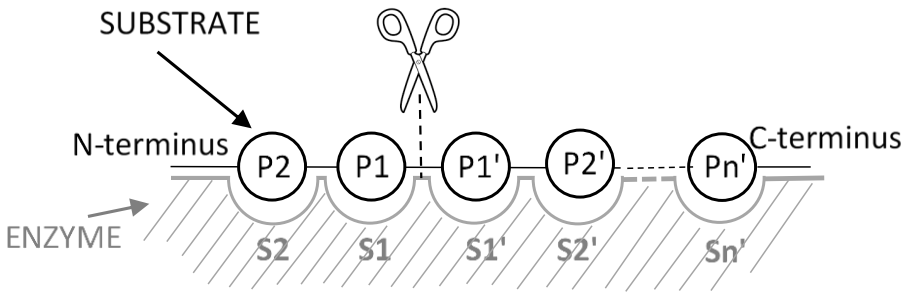
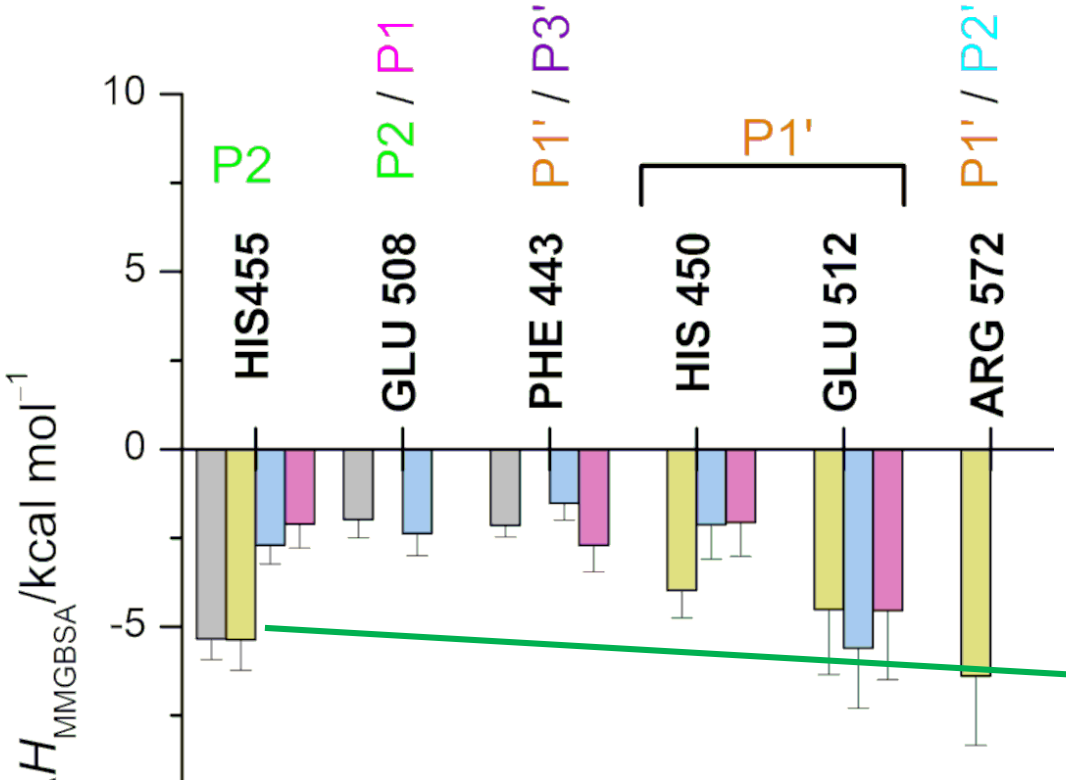
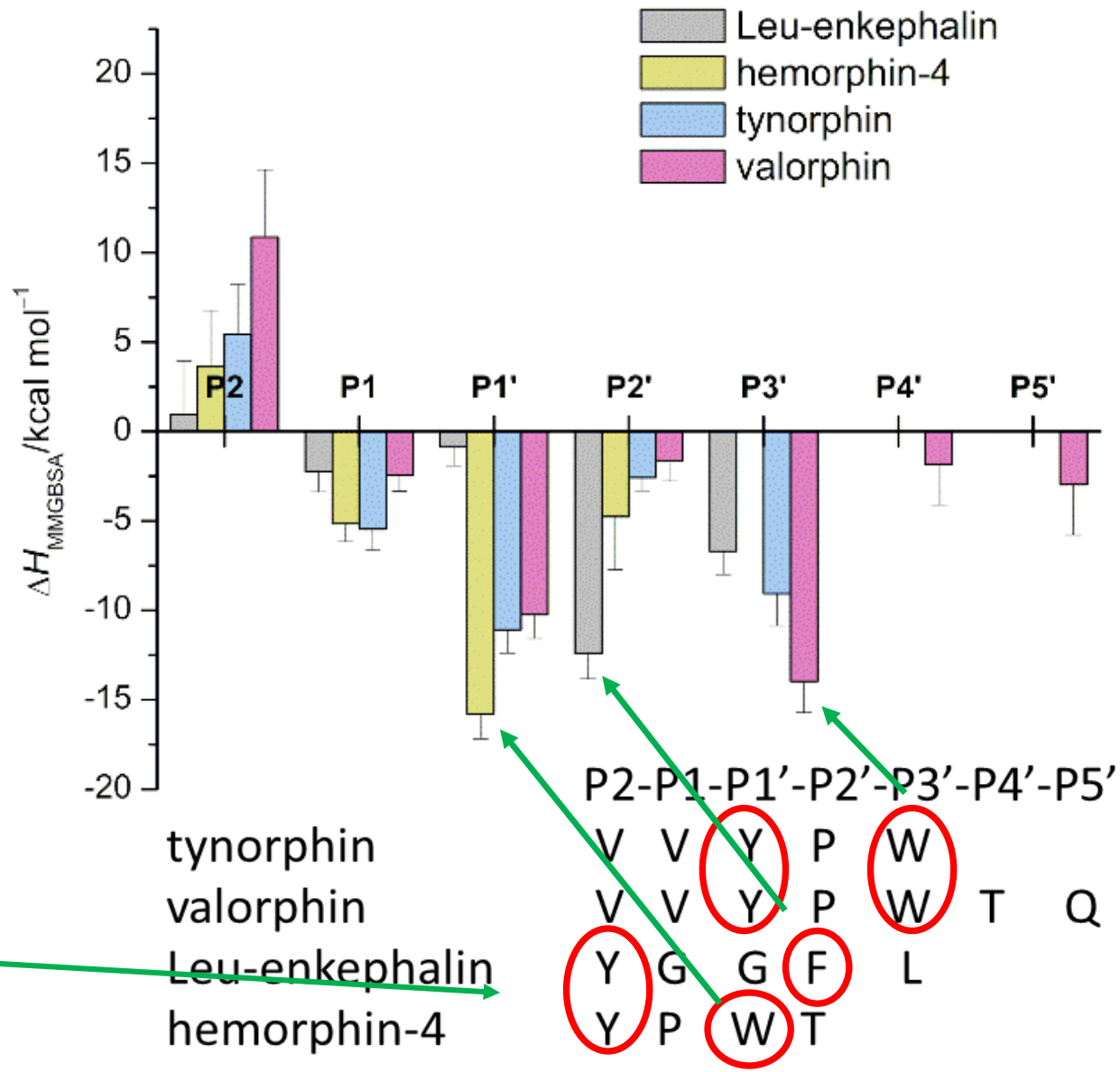
MD simulations

MM/GBSA binding enthalpies calculated for amino acid residues of the protein on a set of conformers sampled during the last 0.6 μs of 1 μs long MD simulations with the lowest binding enthalpies (± SD).

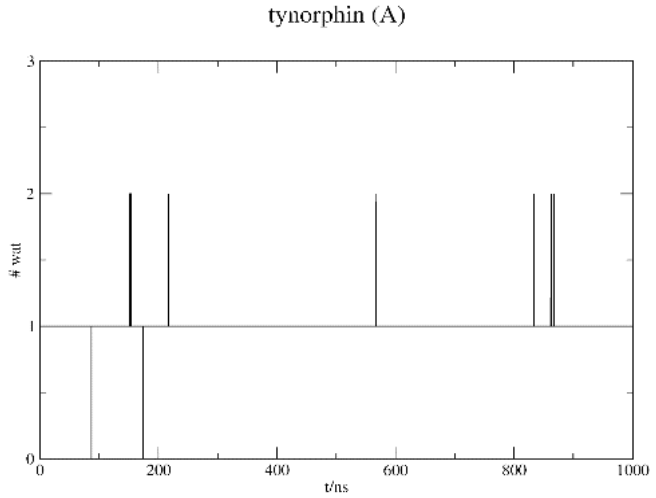


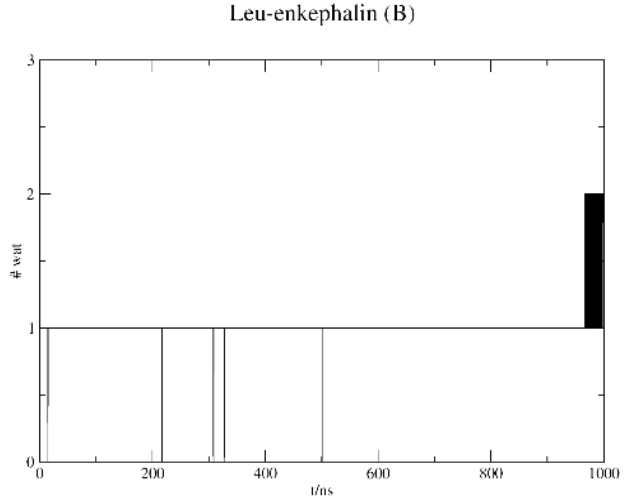


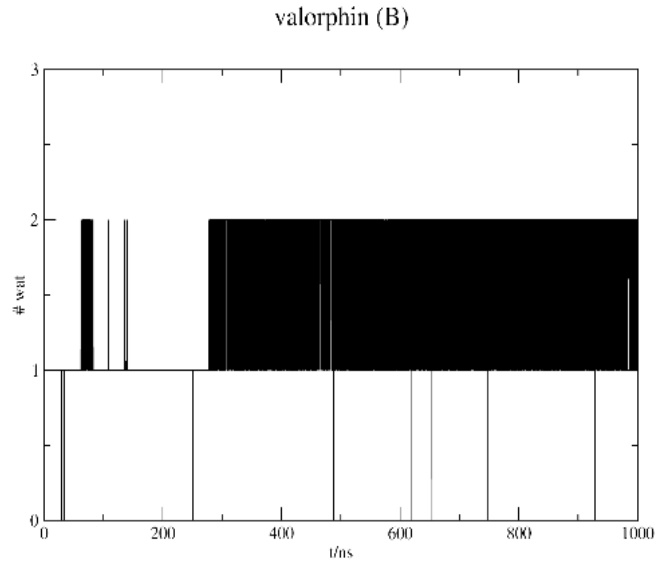


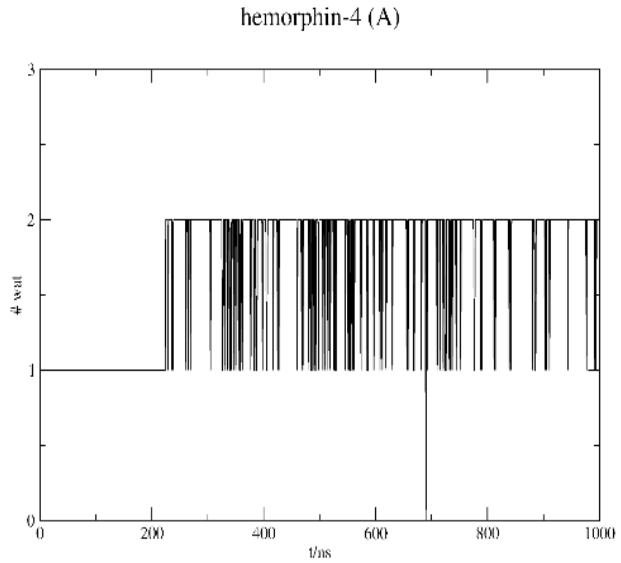
MD simulations

Number of water molecules within 2.5 Å from Zn^{+2} during MD simulations of DPP III in complexes with neuropeptide





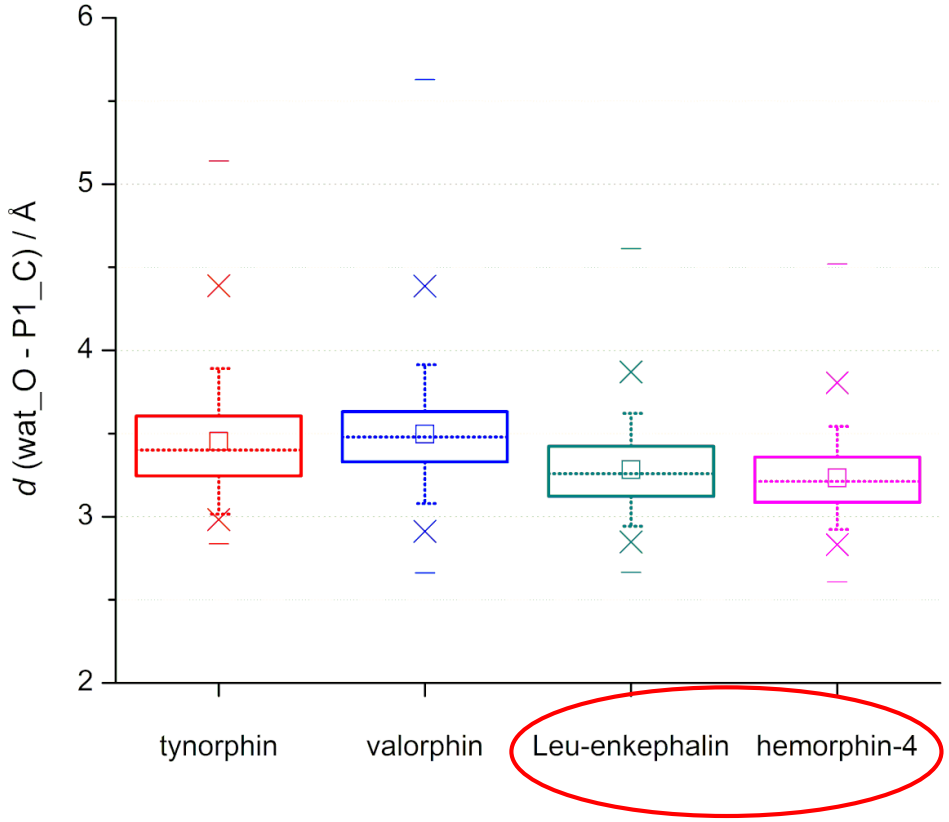




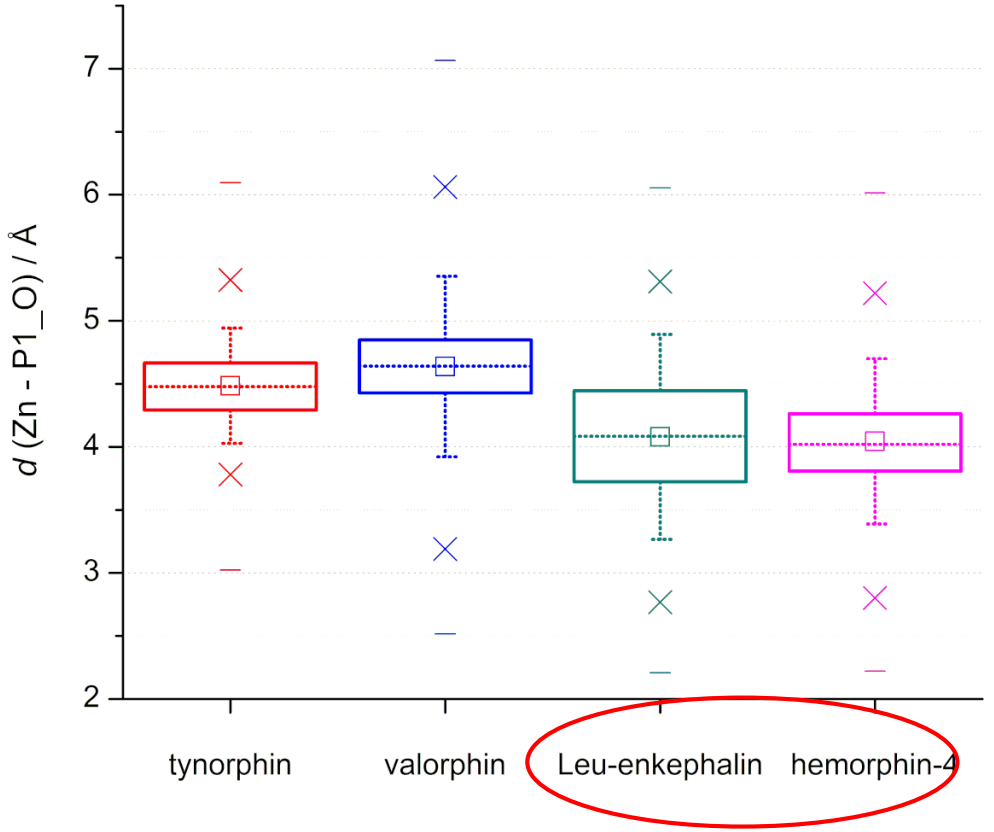
E451

MD simulations

Distances: a) **water O** – carbonyl carbon at P1 position



b) Zn^{+2} – carbonyl oxygen at P1 position



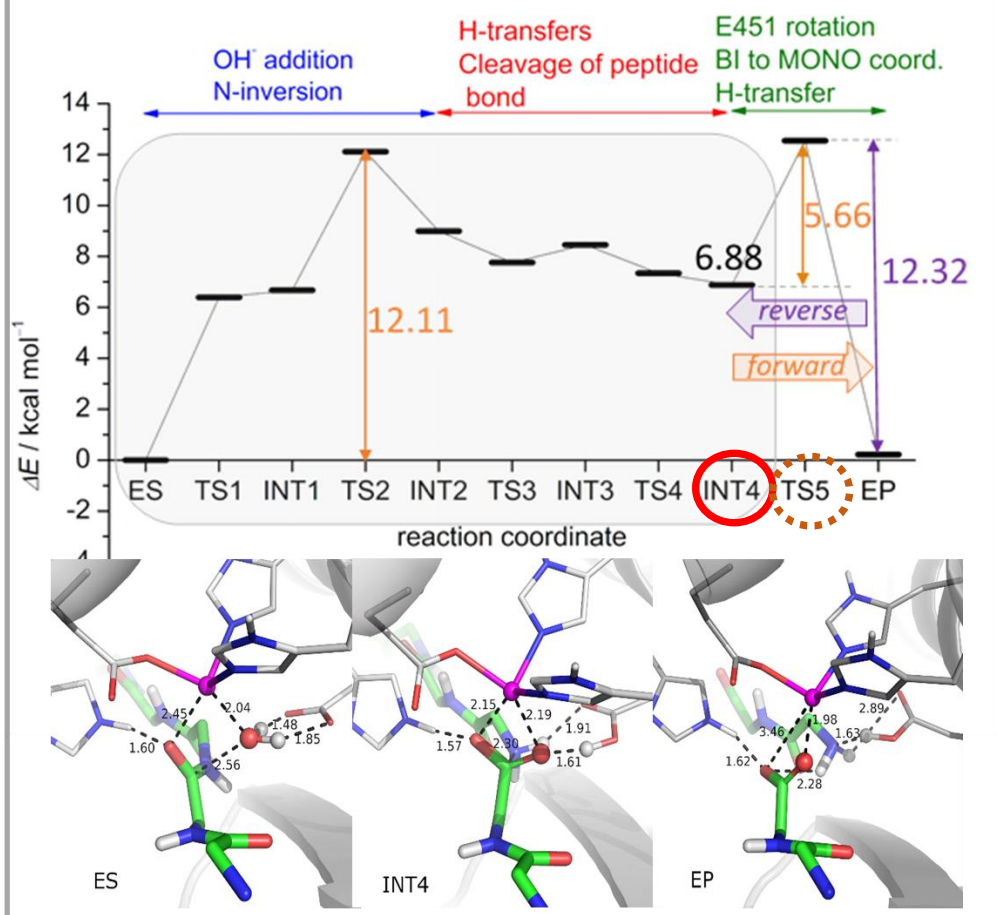
Karačić et al. Neuropeptides, substrates and inhibitors of human dipeptidyl peptides III, experimental and computational study - a new substrate identified, *IJBM*, under review

Mechanism of hydrolysis

QM/MM B97D [6-31G(d) + LanL2DZ-ECP] calculations

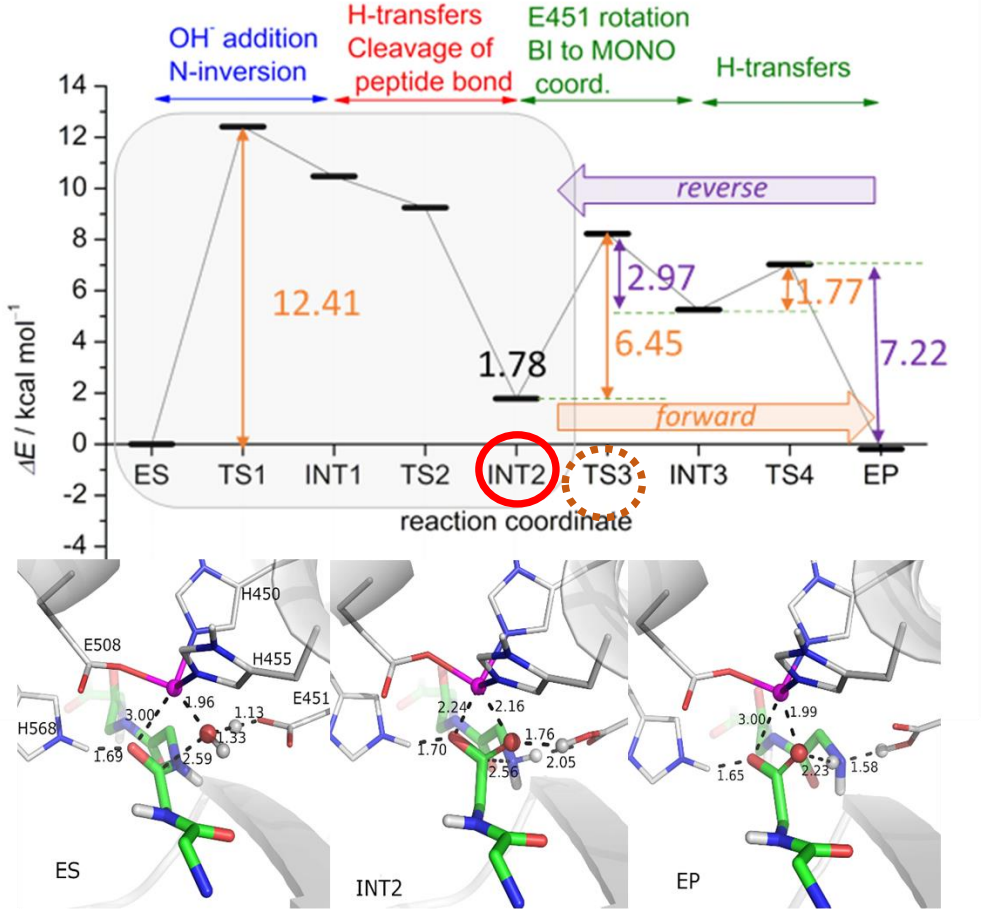
Tyr – Gly ÷ Gly – Phe – Leu

[Leu-enkephalin]



Val – Val ÷ Tyr – Pro – Trp

[tynorphin]



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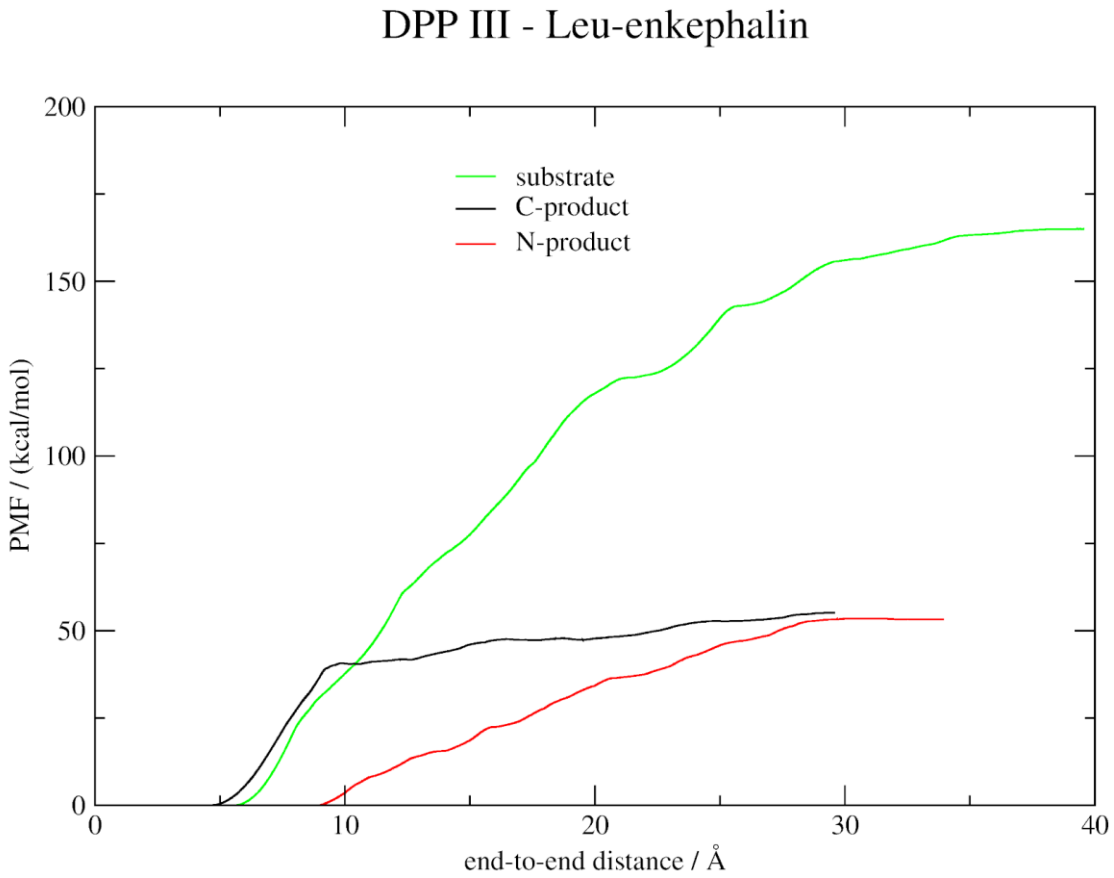
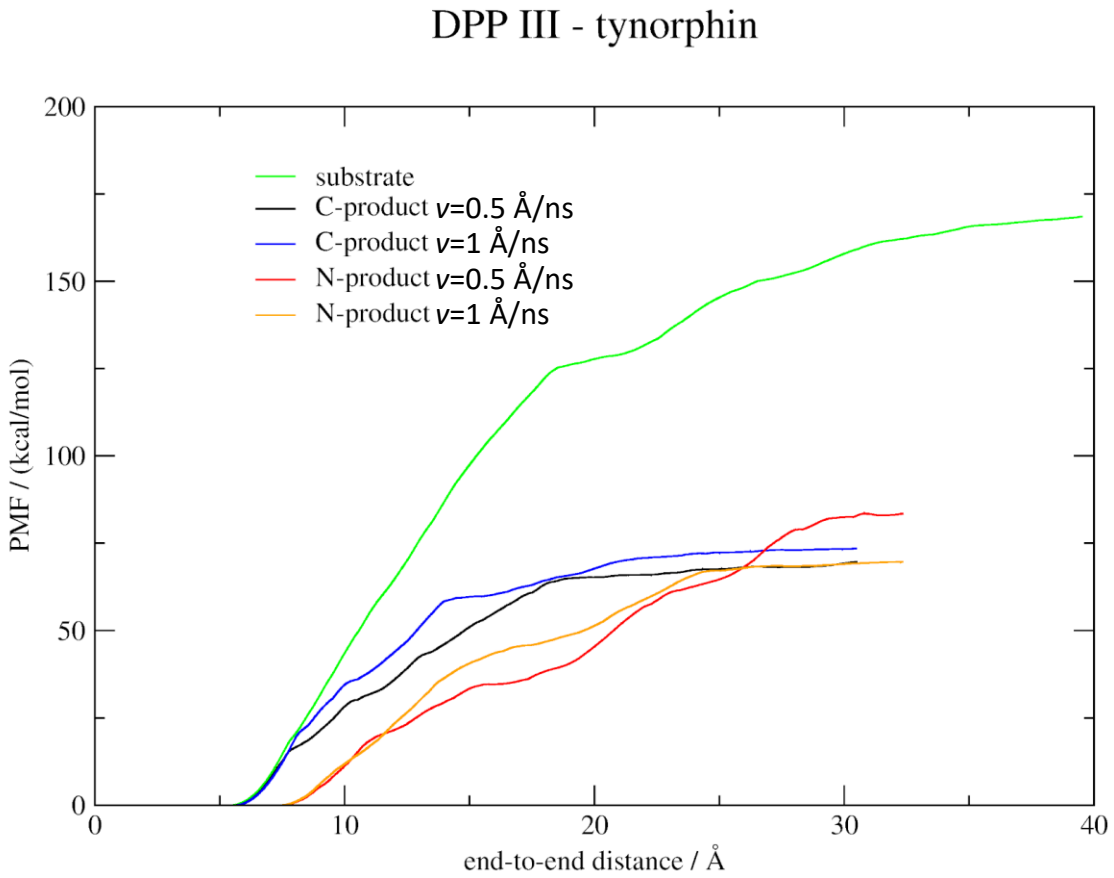
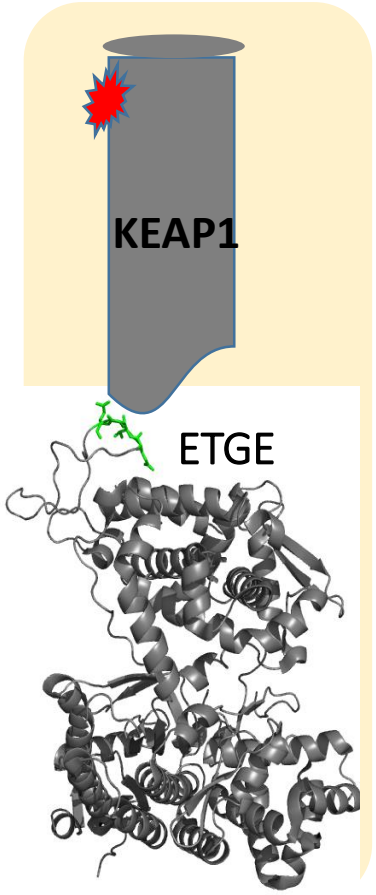
ASMD simulations

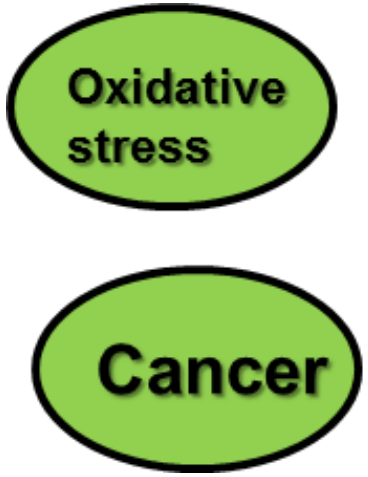
PMF profiles for the release of substrates and products from the enzyme binding site into the solvent environment







Dipeptidyl Peptidase III possible target for improving efficiency of chemotherapy



Increased expression

Increased concentration

Increased activity

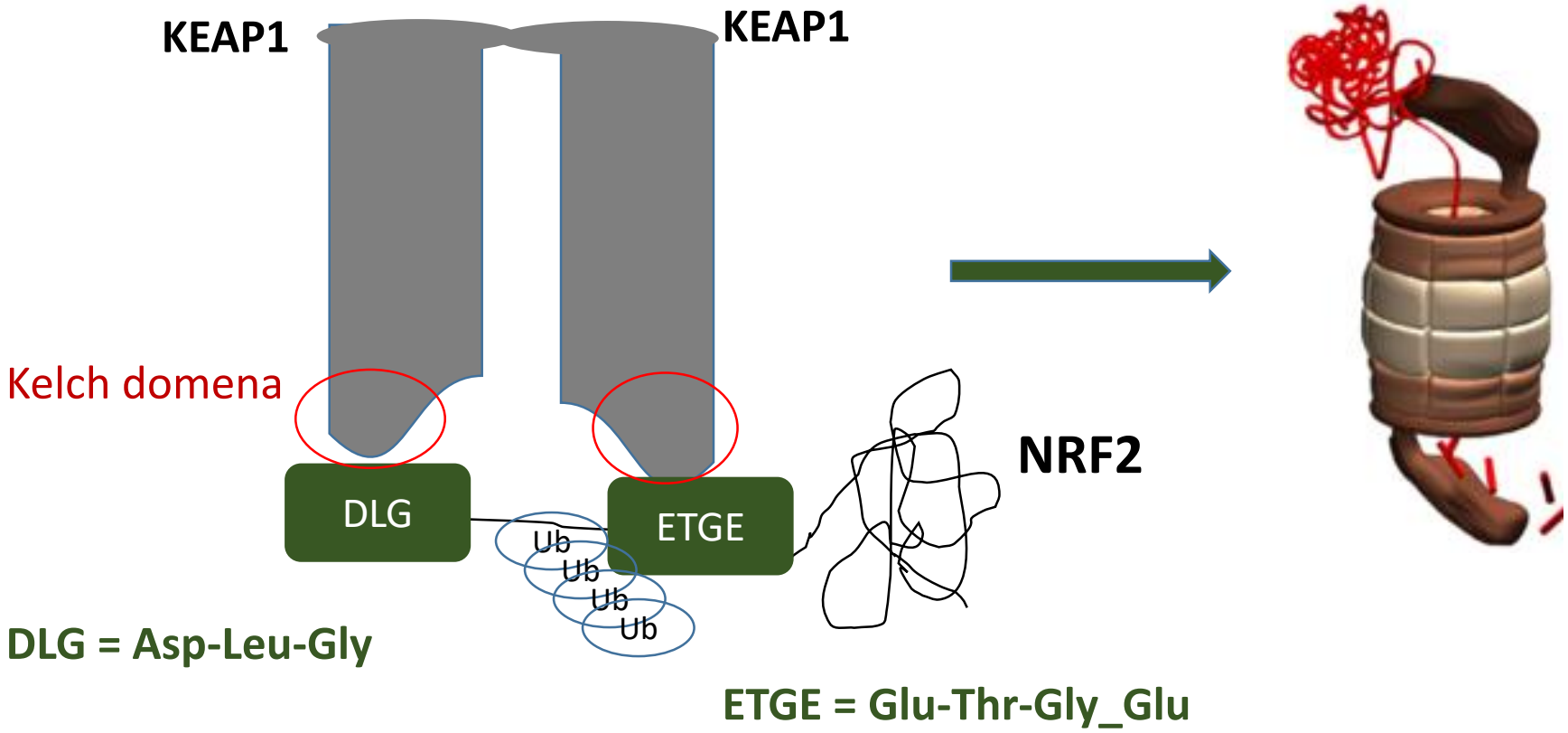
Šimaga et al. (*Eur. J. Cancer* **1998**, *34*, 399) detected increased levels and activity of DPP III in malignant endometrial tissue
Šimaga et al. (*Gynecol. Oncol.* **2003**, *91*, 194) found that expression of DPP III has been positively correlated with ovarian cancer aggressiveness

Gamrekelashvil et al. (*Cell. Mol. Life Sci.* **2015**, *72*, 273) showed that DPP III is epigenetically induced in liver cancer cells by promoter hypomethylation, while DPP III and thimet oligopeptidase-1 (TOP-1) decrease the immunogenicity of necrotic tumor cells by blocking antigen cross-presentation

Miettinen *et al.* (*Cancers (Basel).* **13**, (2021). DOI: 10.3390/cancers13071527) found that **higher expression of DPP III correlates with shorter survival of patients with multiple myeloma**, and the increased level of DPP III in patients with relapsed multiple myeloma compared to newly diagnosed patients suggests that it may be involved in cancer

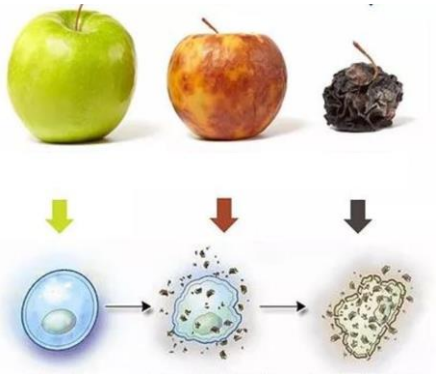
KEAP1-NRF2 – pathway in regular conditions

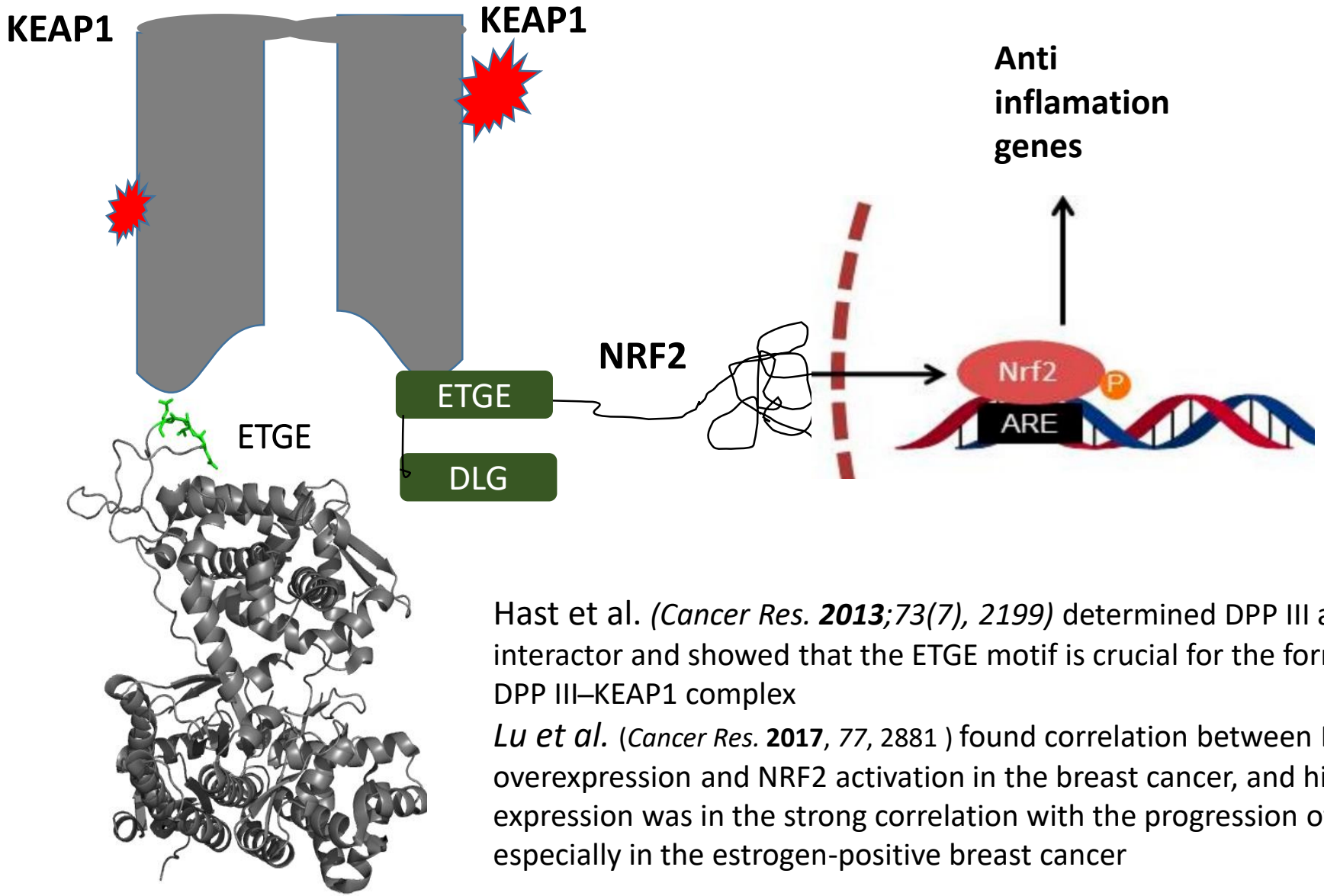
Kelch-like ECH-associated protein 1 (KEAP1) – NRF2 (Nuclear factor [erythroid-derived 2]-like 2 protein)



KEAP1 mediated degradation of NRF2 *via* the ubiquitin-proteosome pathway

KEAP1-NRF2 – pathway under the oxidative stress

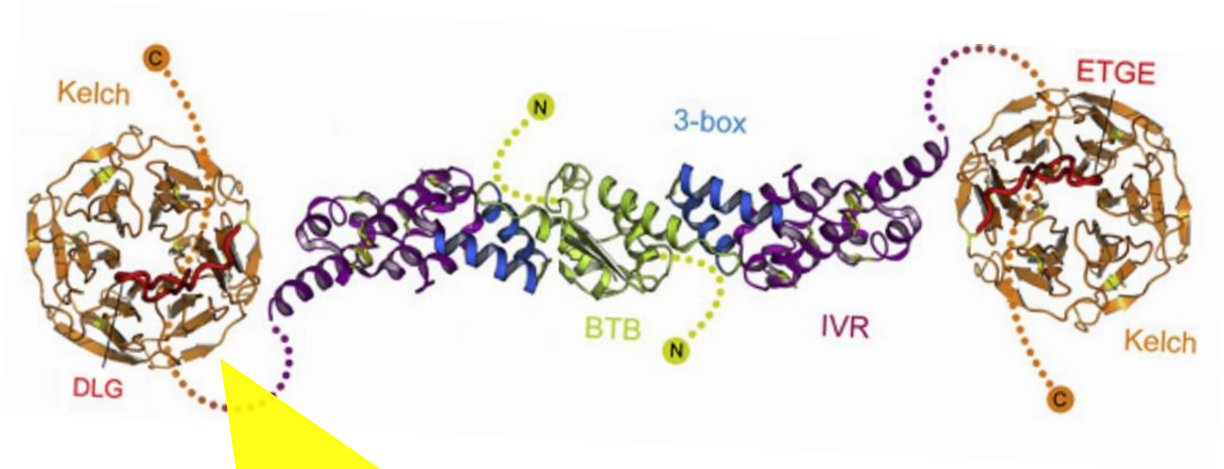


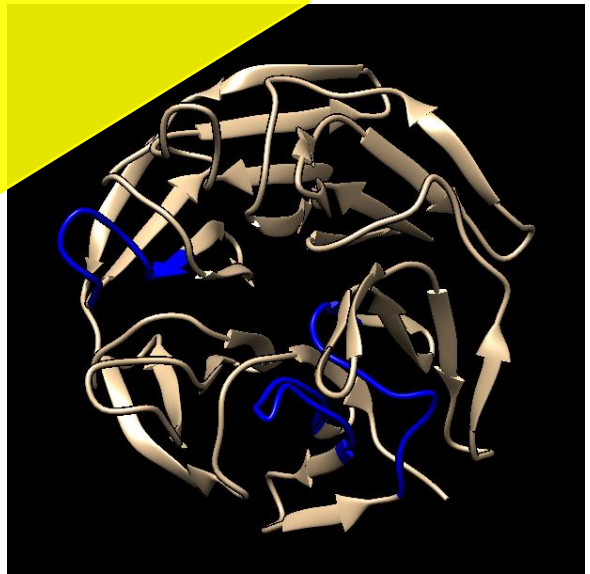


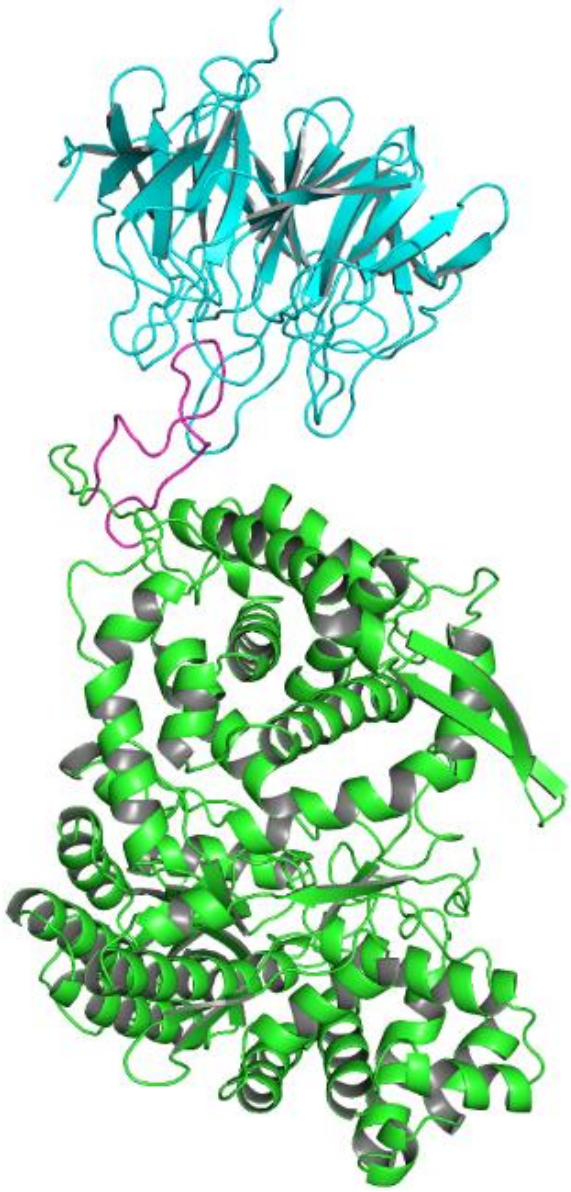
Hast et al. *(Cancer Res.* ***2013****;73(7), 2199)* determined DPP III as a KEAP1 interactor and showed that the ETGE motif is crucial for the formation of the DPP III–KEAP1 complex

Lu et al. (*Cancer Res.* **2017**, *77*, 2881) found correlation between DPP III overexpression and NRF2 activation in the breast cancer, and high DPP III expression was in the strong correlation with the progression of the disease, especially in the estrogen-positive breast cancer

KEAP1-DPP III interactions

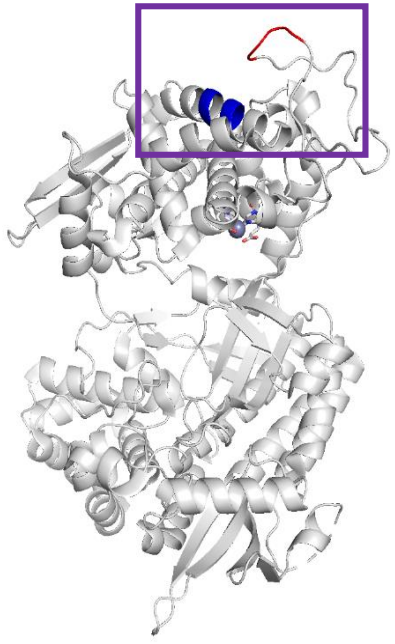


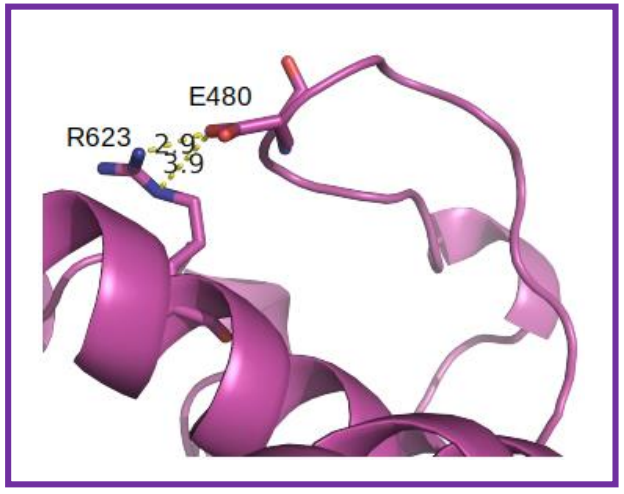


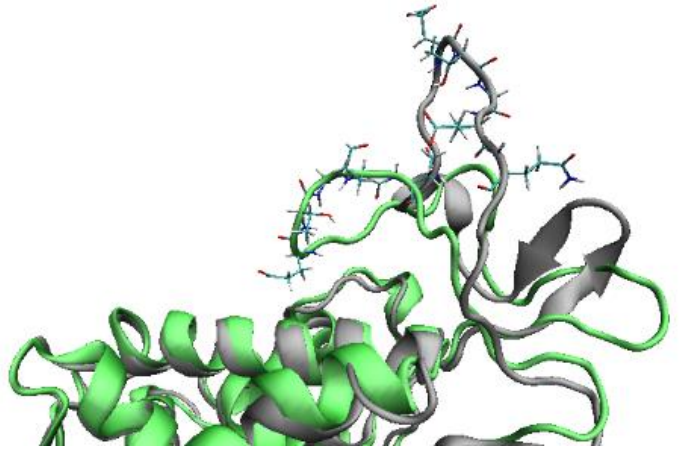


Dipeptidyl peptidase III – Kelch domene

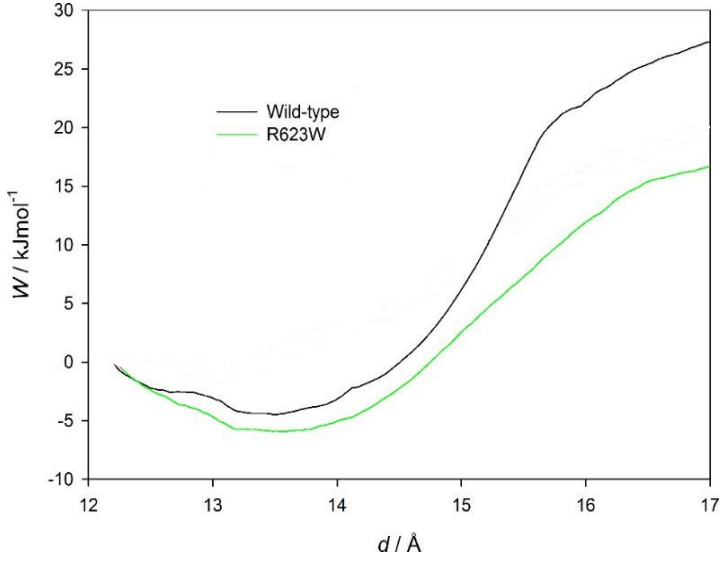
^{480}ETGE^{483} - R620, R623, R624 interactions





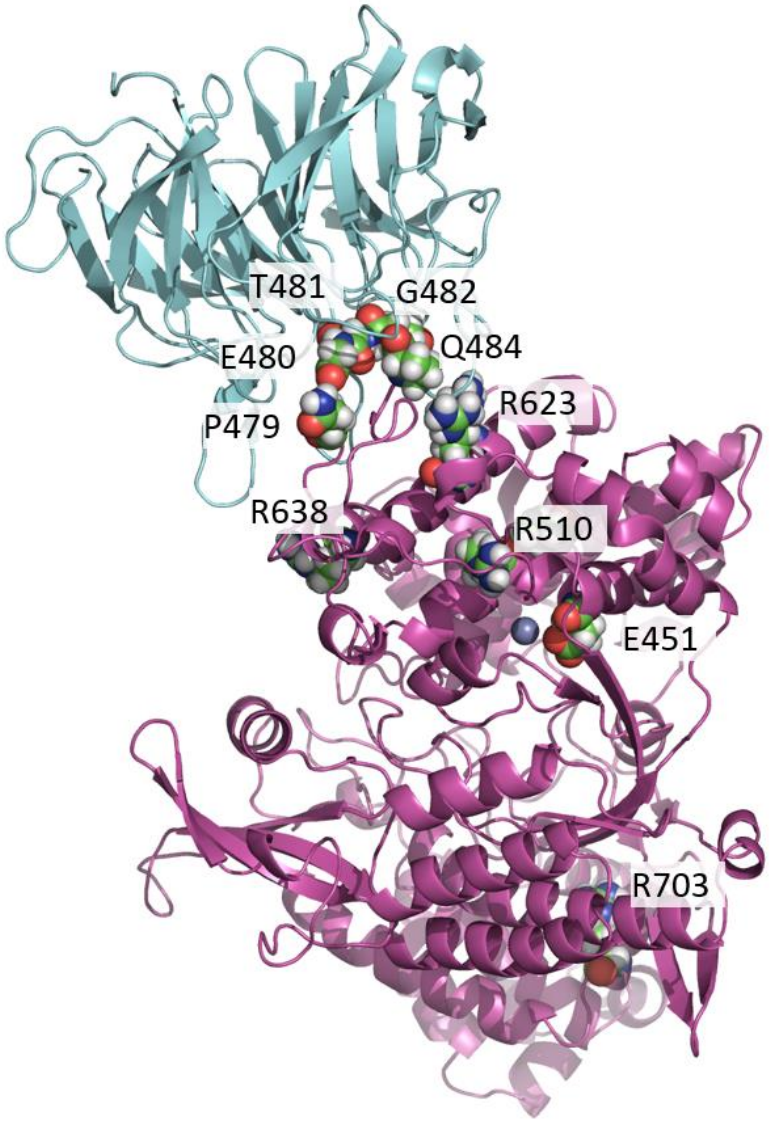


The ETGE motif detachment is an endergonic process



J. Biomol. Struct. Dyn. **2021**, *39*, 6870–6881

DPP III cancer mutations: impact on the affinity for the Kelch domain



MST measurements. Binding affinity of DPP III mutants for the Kelch domain compared to the affinity of the wild-type protein, expressed as the ratio K_d(WT)/K_d(mutant).

DPP III	K_d(WT)/K_d (M)
WT	1.0
P479S	**18.4**
E480Q	**0.1**
T481M	**0.1**
G482C	0.8
Q484H	2.1
R510W	**0.3**
R623W	**160.0**
R638L	**2.0**
R638W	**2.0**
R703C	1.7

K_d of R623W ~ 5×10^{-9} mol dm^{-3}
K_d of WT DPP III ~ 8×10^{-7} mol dm^{-3}

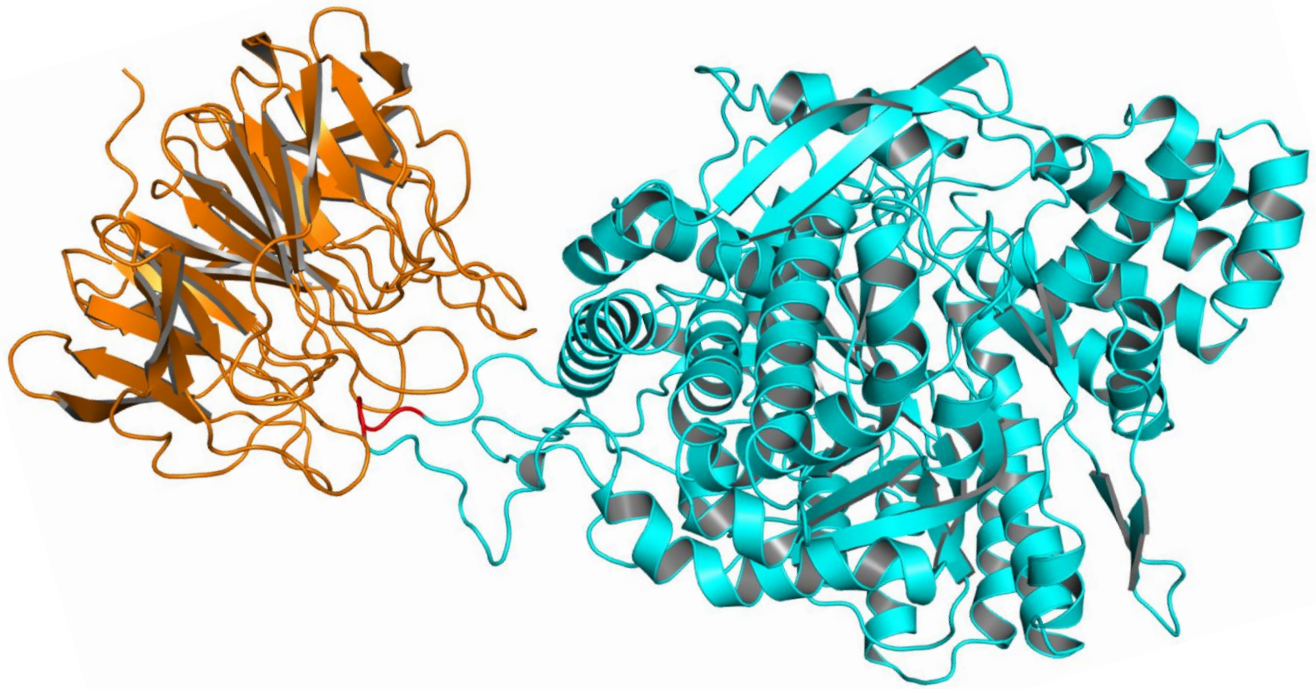
Such a significant increase in affinity for the variant is consistent with our proposed mechanism of binding of DPP III to KEAP1.

Int. J. Mol. Sci. **2022**, 23(4), doi:10.3390/ijms23041994

MM/GBSA energies calculated during 700 ns of MD simulations

DPP III	MMGBSA (kcal/mol)^{min}
WT	**-80**
P479S	**-116**
E480Q	**-42**
T481M	**-63**
G482C	**-56**
R510W	**-69**
R623W	**-65***

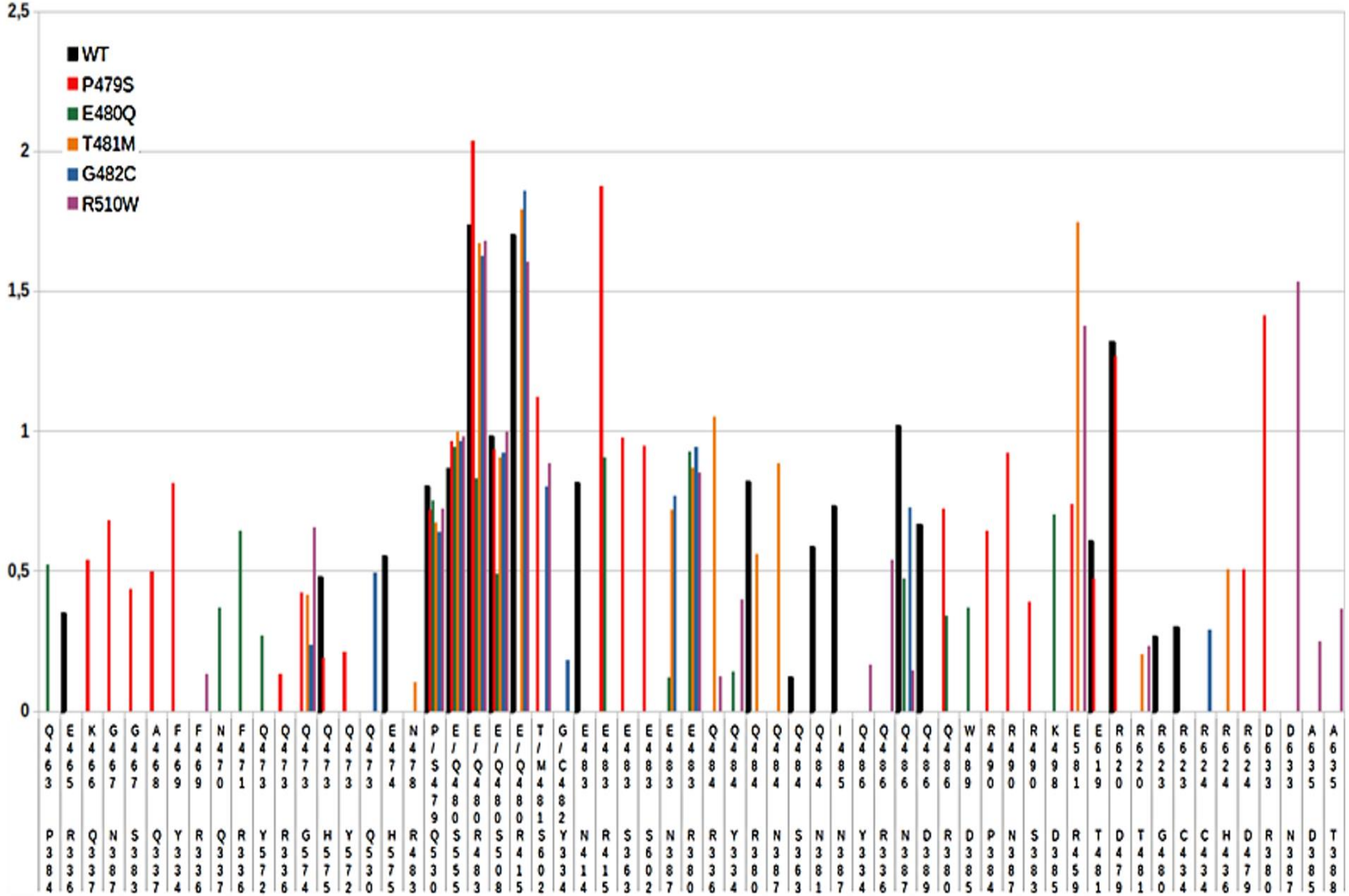
The lowest-energy structure of the DPP III (cyan) – Kelch (orange) complex with the ETGE motif in red.



*The significantly lower binding affinity measured by MST is due to the easier release of the flexible loop from the protein body, the binding affinity increased.

Binding of DPP III to KEAP1 is a two-step process involving endergonic translocation of the loop, followed by exergonic interactions between DPP III and the Kelch domain.

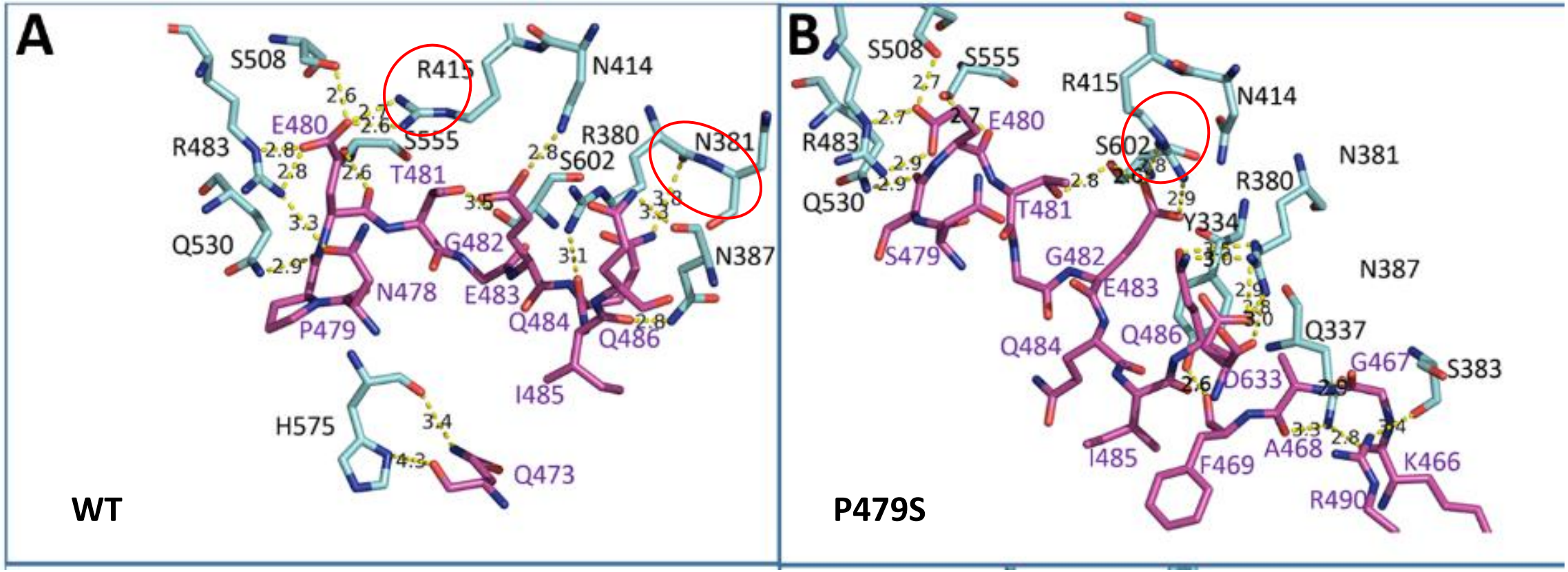
Effect of DPP III mutations on the Kelch – DPP III structure



Distribution of the intermolecular hydrogen bonds determined in the 20 ns long interval with the lowest MM/GBSA energy.

Effect of DPP III mutations on the Kelch – DPP III structure

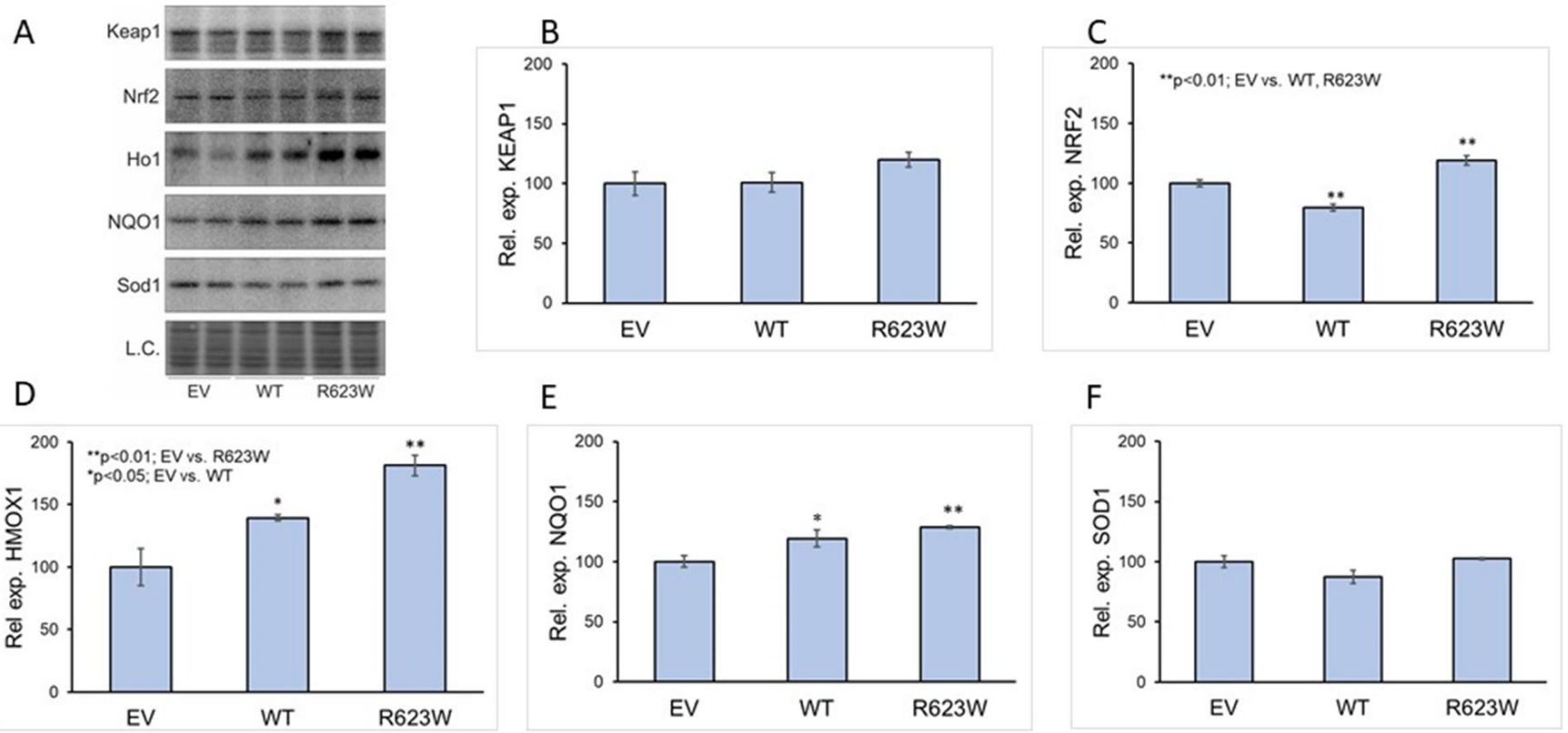
Changes in the intermolecular interactions

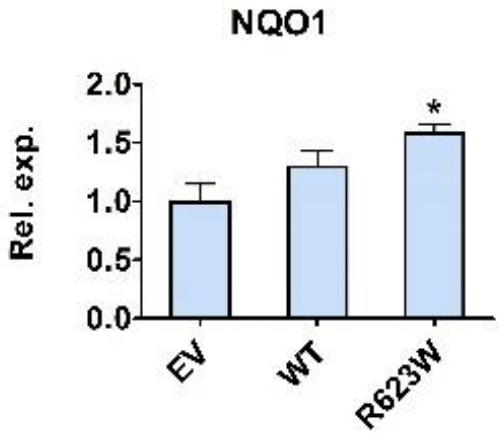


NRF2 EETGE

Effect of DPP III mutations on expression of the NRF2 controlled genes

Western blot analysis of the relative expression of KEAP1, NRF2, HMOX1 (Ho1), NQO1 and SOD1 in the cells transfected with EV, WT and R623W, respectively. R623W was shown to increase the expression of some NRF2-controlled genes.





Relative expression of NQO1 mRNA in HEK293T cells

Summary

- The neuropeptide hemorphin-4 was identified as a new substrate of DPP III, whereas valorphine, Leu-valorfin-Arg, and β-casomorfin, previously identified as inhibitors of DPP III, were found to be its substrates.

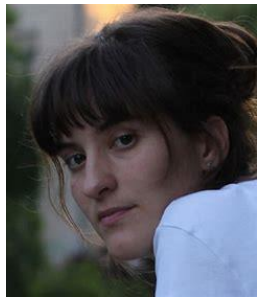
- The enzymatic cycles for the hydrolysis of Leu-enkaphalin and tynorphin by DPP III were determined, and it was found that tynorphin is an inhibitor because of pronounced stabilization of its products in the enzyme active site.

- The influence of different mutations of DPP III, present in human cancer, on the affinity of DPP III for Kelch was investigated, and it was found that the R623W and P479S mutations significantly improved this affinity. The R623W mutation was found to increase the expression of some NRF2 regulating genes in the cell.

Acknowledgement

Sara Matić

Zrinka Karačić



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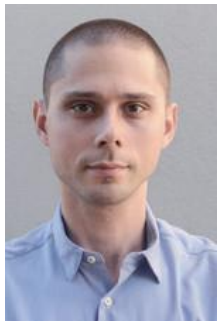

Filip Šupljika

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This work has been fully supported by Croatian Science Foundation under the project IP-2018-01-2936

Thank you!

Questions?

Sugestions?