Prof. Matthias Bochtler

International Institute of Molecular and Cell Biology &
Trojdena 4, 02-109 Warsaw, Poland
Institute of Biochemistry and Biophysics
Pawinskiego 5a, 02-106 Warsaw, Poland
Tel: 0048 22 5970732
Fax: 0048 22 5970715
e-mail: mbochtler@iimcb.gov.pl

Warsaw, March 1st 2022

Opinion on the habilitation thesis by Dr. Dorota Zurawa-Janicka

Dr. Zurawa-Janicka is a biochemist with a research interest in proteases. Her habilitation work revolves around HtrA proteins, the mammalian homologues of the bacterial DegP peptidase. *E. coli* DegP is a very well-studied periplasmic serine endopeptidase. Unusually for a peptidase it also has a chaperone function. The architecture of DegP is also somewhat unusual for peptidases, with two PDZ domains in addition to the catalytic domain serving as an affinity module. This basic architecture is preserved in the human homologues (termed HtrA1-HtrA4). All four paralogues have the catalytic domain, and one (HtrA1 and HtrA2) or two (HtrA3 and HtrA4) PDZ domains. In line with the periplasmic nature of DegP, three of the human HtrA paralogues have a secretion signal in their amino acid sequence. The exception is HtrA2, which instead has a mitochondrial targeting signal and is active in mitochondria. Apart from the core catalytic and PDZ domains, the mammalian HtrAs contain additional domains, including a Kazal-type inhibitor motif (HtrA1 and HtrA3), and an IAP binding domain. The latter domain is a give-away on function.

Inhibitors of apoptosis (IAPs) promote survival by inhibiting apoptotic caspases. The ability of HtrAs to bind and presumably degrade inhibitors of apoptosis suggests that the protein may act as pro-apoptotic factor. As clearly laid out in a 2010 review (Expert Opinion on Therapeutic Targets (2010) 14: 665-679, **publication 1**), an anti-apoptotic role is indeed confirmed by the observation that HtrAs are frequently either lost or silenced in malignancies, which may help a tumor with its survival. Decreased survival as a result of HtrA action appears to be not only a consequence of IAP degradation. Dr Zurawa-Janicka argues convincingly that a part of the effect is due to downregulation of transforming growth factor β (TGF β) signaling. It has often been argued, not only by Dr. Zurawa-Janicka in her reviews, that HtrA proteins make good targets for cancer therapy. I am somewhat skeptical about this claim, since any HtrA directed small molecule would have to be activating, rather than inhibitory. Moreover, when HtrA is fully silenced or lost in tumors, no small molecule could possibly bring the its activity back. That aside, the asset of HtrAs as drug targets lies in their at least partly extracellular nature. Hence, drug delivery should be somewhat easier, and any potential small molecule agents would not have to be cell permeable. It is unclear, however, to what extent the proapoptotic properties of the HtrAs depend on the extracellular fraction (substrates like TGF β), and to what extent the intracellular fraction plays a role (substrates like IAP).

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Two of Dr. Zurawa-Janicka's research publications are directly related to the role of HtrA proteins in cancer. One (Oncology Reports (2012) 28: 1838-1844, **publication 2**) addresses the expression of HtrA1, HtrA2 and HtrA3 in benign and malignant thyroid tumors. Based on what Dr. Zurawa-Janicka explains very well in her review on the role of HtrA proteins in cancer, one may expect that a lower expression of the HtrA proteins may benefit the tumor, whereas overexpression should be bad for the tumor. Paradoxically, however, the opposite is found. In Dr. Zurawa-Janicka's own words, the findings may be summarized as "The levels of HtrA2, HtrA3-S and HtrA3-L are increased in thyroid cancer." Although the work is published after the very clear review on HtrAs in cancer (publication 1), the Discussion does not really address the discrepancy. And looking at the raw data, the reader remains unsure whether thyroid malignancy really is an exception to the proapoptotic role of the HtrAs. Inspection of HtrA levels in individual patients or controls shows that the variations within groups is larger than the average variation between groups. Moreover, there is a suspicion that a single outlier with very high HtrA expression (in case of HtrA1, Fig. 1 of publication 2) may unduly influence statistics, which have (unmet) assumptions on underlying data distributions. In short, I would be more comfortable with the conclusions of this study if the cohorts were larger, and if a clustering analysis would show that the controls and tumor samples are really forming separate clusters. Otherwise, there remains a nagging suspicion that the data may not be significant (even if p-values are acceptable, but note the borderline significance for HtrA1 in Table 1 of publication 2), especially when there is no good reason why data deviate from the "big picture".

The other of Dr. Zurawa-Janicka's directly cancer-related publications (Int. J. Mol. Sci. (2020), 21:3947, **publication 7**) addresses possible correlations of HtrA gene expression with microsatellite instability and colon cancer. And in this setting, the pro-apoptotic effect of the HtrAs is seen. HtrA protein levels are lower in the tumors than in the controls. In contrast to the thyroid study, there also appears to be more consistency between groups. Interestingly, the qPCR quantification suggests that this effect is not related to mRNA levels, which are either very similar in tumors and controls, or even higher in tumors compared to controls. The implication is that the effects must either stem from decreased translation in the tumors or from more rapid degradation of the HtrA proteins in the tumors. This may be an interesting lead for further studies. As one may expect from the broad picture of HtrA proteins in cancer, a high level of HtrA1 or HtrA2, or of both proteins together, is predictive of better survival. In this work, which I regard as perhaps the best of the series of habilitation publications, Dr. Zurawa-Janicka also reports correlations of HtrA levels with microsatellite instability. The finding is that a decrease in the HtrA1 and HtrA2 transcript levels is associated with higher microsatellite instability. This is plausible, since a low HtrA1 and HtrA2 level is "good for the tumor" and may be predictive of a more advanced stage of the malignancy.

The reminder of Dr. Zurawa-Janicka's publications address more mechanistic questions of HtrA biology.

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I will start my comments with the review on the topic (Archives in Biochemistry and Biophysics (2017) 621: 6-23, **publication 6**). In this excellent and balanced review, Dr. Zurawa-Janicka and co-workers first describe domain organization of the HtrA proteins and their relevance before focusing on the still confusing regulatory mechanisms. For the three described HtrA paralogues (HtrA1, HtrA2, HtrA3), the basic organization into trimers, mediated by the protease domains, is conserved. The allosteric activation of the active site seems to be another common theme in all three proteases. The HtrAs differ in the role of the PDZ domains. They are surprisingly dispensable in case of HtrA1, and have an unclear role in HtrA3 (only one of three PDZ domains is in a defined orientation in the crystal structure). By contrast, there is a relatively clear and plausible model for HtrA2. For this protein, it seems that the PDZ domains can adopt a closed conformation that blocks active sites, and an open conformation that leaves active sites accessible. Given Dr. Zurawa-Janicka's assessment of HtrA2 as the most relevant target for drug development, and given the prospect for a "clean" activation mechanism, it was reasonable to focus mechanistic work on HtrA2.

In the first mechanistic publication (Cell Stress and Chaperones (2013) 18:35–51, **publication 3**), Dr. Zurawa-Janicka looks at temperature mediated activation of HtrA2, using tryptophan fluorescence as a technique. More specifically, the emission maximum wavelength is used as a reporter for the hydrophilicity or hydrophobicity of the tryptophan environment. As Dr. Zurawa-Janicka is interested PDZ repositioning in the regulation of HtrA2 activity, the tryptophan probes are mostly placed at the interface between the protease and PDZ domains. As expected, a loosening of the structure, indicated by the more polar environment of the tryptophan residues, is observed. Unfortunately, this is a quite generic result that would be expected for most proteins. Moreover, thermal activation of enzymes is likely to be a complex combination of several effects. In the case of HtrA2, increased probability for achieving a transition state (exp(-kT/E), where E is the activation energy), active site loop opening, and PDZ domain conformation can all be expected to play a role in the increa of activity at higher temperature. From my perspective, a more specific activation mechanism (for example by a substrate peptide), and a reporter system more selectively probing domain conformation (for example, by Foerster transfer) would have been preferable.

In the second mechanistic publication (Biochimica et Biophysica Acta. Proteins and Proteomics (2016) 1864: 283-296, **publication 4**), Dr. Zurawa-Janicka and her colleagues look again at thermal activation of HtrA2, but this time, they focus on intra- and intersubunit changes. The key technique here are time-resolved tryptophan fluorescence and tryptophan induced quenching. The latter is an alternative to Foerster resonance energy transfer (FRET). In contrast to FRET, which works optimally for distances in the range ~20 – 100 Å, it is more suited to shorter length-scales ~5 – 15 Å. Somewhat regrettably, again thermal activation is used instead of substrate induced activation as a trigger for changes. The key conclusion from the work is that upon activation PDZ domains change orientation with respect to protease domains (both within the same and in separate chains). The results are very reasonable, but to some extent expected. My main

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reservation here is that it is unclear to what extent thermal activation recapitulates activation by the presence of a substrate.

Dr. Zurawa-Janicka and co-workers have also made a considerable effort to test the HtrA activation mechanism in atomic detail (PloS One (2016) 11: e0161526, **publication 5**), by X-ray crystallography. The general concept here is that in the "resting" state, the HtrAs are arranged so that there is a tight complex between the protease and PDZ domains. It is further thought that binding of peptides to the PDZ domain displaces the protease domain, so that the open conformation of HtrA is able to interact with the target and ultimately cleave it. Structures of HtrAs in closed conformation were already available prior to the work of Dr. Zurawa-Janicka. The authors hoped to trap an open conformation by co-crystallizing an inactive variant of HtrA2 (S306A) with a peptide substrate. Their experimental design included the choice of a hyperactive variant (V226K) that was reasonably assumed to stabilize the open conformation. The authors got diffracting crystals and found -what must have been an immense disappointment- that they contained the HtrA2 in the closed conformation. To the authors disadvantage, compact molecules are much more likely to crystallize than molecules with flexible hinges. My interpretation of the data is that the growth of diffracting crystals effectively selects for the closed conformation and thus this conformation will be crystallized, no matter the conformational equilibrium in solution. The authors rescued the situation with relatively long (50 ns) molecular dynamics studies, but this is no equivalent for an experimental structure, especially when molecular dynamics had to be "steered". I understand that this was necessary here to access events that are otherwise too slow to be observed, but the steering always means that the results are biased by the prejudice of what motions should be expected.

Having been myself judged very harshly for not publishing in sufficiently highly ranked journals, I am in an awkward position to criticize Dr. Zurawa-Janicka for the moderate impact of journals (all < 6, many < 3) that she has published in. Moreover, I see that journal impact might be a poor measure of the relevance of her work in some cases, especially when the actual work is well cited (as for example publication 1). Nonetheless, I would advise Dr. Zurawa-Janicka to aim higher in the future, to avoid trouble with supervisory committees and advisory boards.

I could see a few ways for Dr. Zurawa-Janicka to publish higher. So far, most of her publications, with the notable exception of publication 7) describe the results from just one or two methods. Nowadays, such work is hard to publish for all of us. Therefore I would suggest to combine results from more diverse methods into single publications. Also, some of her papers are very technical, which editors are not keen on. Perhaps the publications could be made more accessible by relegating much of the technical information to Suppl. Materials, to be able to write a "cleaner" and "slimmer" and therefore more appealing main text. Finally, there is the issue that in some cases, Dr. Zurawa-Janicka's publications run counter to the broad themes in

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the field (particularly publication 2). There is nothing wrong with challenging the orthodoxy, but one should either know what was wrong with the orthodoxy, or alternatively, one should have a clear rationale why the orthodoxy is not applicable in a special case. Without either, the reader is left wondering about whether the results may be the consequence of a statistical quirk, or some systematic bias, especially when conclusions rely on a single method only (a particular concern for publication 2). Finally, in some cases, the reader gets the impression that mostly negative data are present (publication 5, the closed conformation of HtrA2 in the crystals).

Despite these reservations, I see the work positively. The publications are very focused on one coherent theme that Dr. Zurawa-Janicka has concentrated on consistently for many years. Despite the relatively moderate impact of journals that she has published in, the overall citation statistics for Dr. Zurawa-Janicka (H-factor 14, 847 citations) are good for this career stage. Working at a university and not a dedicated research institute, she has had extensive teaching commitments that must have taken up a considerable amount of her time. Hence, I conclude **that the habilitation thesis by Dr. Zurawa-Janicka meets the requirements laid out in the law nr 219, paragraphs 1-3,** in Polish: **"Oświadczam, że rozprawa habilitacyjna dr Żurawy Janickiej spełnia wymagania, o których mowa w art. 219 ust. 1 pkt 1-3 p.s.w.n"**

With best regards

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