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Opinion on the PhD thesis by Valeria Napolitano

Valeria Napolitano's PhD thesis "Targeting the glycosomal protein import machinery: new strategies against Trypanosomiasis" describes a medical chemistry effort to target the glycosome import machinery of trypanosomatids, the causative agents of Chagas disease (caused by *Trypanosoma cruzi)*, African sleeping sickness (caused by *Trypanosoma brucei)*, and leishmaniasis (caused by *Leishmania* protozoan parasites). While African sleeping sickness is on the decline (and may be eradicated by the WHO efforts in the foreseeable future), the other two diseases remain very much relevant. Moreover, as Valeria Napolitano's PhD thesis points out, the pipeline for new drugs is thin. If the few candidates currently in clinical trials fail, there will be no new drugs for at least a decade.

With the rationale for the target convincingly explained, Valeria Napolitano next makes the case for targeting glycosomes. The case is strong indeed: for eukaryotic parasites, it is always difficult to distinguish between host and target. Hence, any features that are unique to the target are worth exploring. Glycolysis is a good example. In trypanosomatids, it is compartmentalized in glycosomes, whereas it takes place in the cytoplasm in humans. Hence, blocked protein import to the glycosomes should be selectively toxic to the trypanosomatids. The rationale is strongest in the case of *T. brucei*, which relies entirely on glycolysis for ATP generation in the blood-stream form. Interestingly, however, inhibition of protein import to glycosomes seems to be toxic not only by the loss of function. Valeria Napolitano argues convincingly that the failure to import glycolysis pathway enzymes to glycosomes leads to their accumulation in the cytoplasm, which is toxic because the regulatory features that prevent toxicity in humans are missing from the parasite enzymes. The "gain of toxic function" mechanism gives strong hope that the glycosome targeting drugs could also work for parasites that unlike *T. brucei* are not strictly dependent on glycolysis.

With the case for targeting parasite glycosomes made, Valeria Napolitano can address the next difficulty. Glycosomes are absent from humans, but they are still related to peroxisomes, which are present in humans, and use similar import machinery. Inhibitor design or optimization is therefore always a task with a target and an anti-target. This is taken well care of in the entire work. Cellular assays on trypanosomatid cells are coupled with toxicity studies on human cells.

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As molecular targets to hinder glycosomal import, Valeria Napolitano has chosen the interfaces between PEX14 and PEX5, and the PEX5-PTS1 interaction. To understand this choice, it is useful to recall the biogenesis of glycosome proteins (see Fig. 3 of the thesis). They are synthesized in the cytoplasm, with a targeting signal termed the PTS1. This signal is recognized in the cytosol by PEX5, which has affinity to PEX14, the actual glycosome importer.

Already prior to the work of Valeria Napolitano, inhibitors of the PEX14-PEX5 interaction were known, including an optimized sub-micromolar inhibitor of the pyrazolo[4,3-c]pyridine class of compounds. The inhibitor binds to PEX14. With the structure of the complex, Valeria Napolitano solved the first structure of a sub-micromolar PEX14-PEX5 protein interface inhibitor with trypanocidal activity. Analysis of the affinity of a series of related compounds revealed the often overlooked contribution of water molecules in the interaction of ligands with their hydrophobic receptor. Otherwise hard-to-explain trends in binding constants could be convincingly interpreted by showing that the more tightly binding inhibitors were simply better at displacing "unhappy" (in the diction of the thesis) surface water molecules in the cavity. While the principle is based on the well-known hydrophobic effect, it was new to me that the position of such water molecules in the empty cavity can be predicted confidently, so that docking of inhibitors and analysis of displaced waters becomes predictive (or at least retrospectively explanatory) for ligand affinity. My (very minor) comment to this part is that I would focus less on "energy", as Valeria Napolitano does. Instead, I would emphasize the large entropy gain associated with the release of water molecules from a hydrophobic surface into bulk solvent, which is partly offset by enthalpic cost. This cost is larger for "happily" bound than "unhappily" bound molecules. Valeria Napolitano's statements are fine, provided one reads "energy" as "Gibbs free energy".

For reasons that are not detailed in the thesis, the pyrazolo[4,3-c]pyridine class inhibitors appear to have finally hit a dead-end in further development. Therefore, Valeria Napolitano carried out a high-throughput screen for other inhibitor scaffolds, and found compounds with a scaffold reminiscent of the dibenzooxacepin with additional heteroatoms (sulfur, nitrogen) in the rings. After pharmacokinetic studies identified the heteroatoms as a liability, more compounds with them replaced by carbons were synthesized. The compounds were further optimized by varying regions of the molecule projecting either into the "so-called" tryptophan pocket (R2), or into the solvent exposed region (R1). For some of the synthesized compounds, binding pose was confirmed crystallographically.

In addition to the work on the PEX14-PEX5 interface, Valeria Napolitano also carried out work on another potential target, the PEX5 receptor region for the PTS1 targeting signal. From bioinformatic work alone, it was already clear that the PTS1 binding region of PEX5 consists of tetratricopeptide repeats. As a basis for the drug design work, Valeria Napolitano solved a co-crystal structure of the domain with a PTS1-containing peptide. In parallel, she set up a fluorescence polarization assay for high-throughput screening of the PEX5-

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PTS1 interaction inhibitors. The top hits were then cross-validated by ^{1}H-^{15}N HSQC experiments and the AlphaScreen assay.

The spectrum of methods that has been applied in Valeria Napolitano's PhD thesis is impressive. She reports two screens for lead compounds. One is based on an elegant high throughput assay for protein interactions called the "AlphaScreen assay". This proximity assay is not based on Foerster transfer, but instead on the diffusion of oxygen radicals. The other assay is the more conventional fluorescence polarization assay. Occasionally, microscale thermophoresis is also used. ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) NMR was used to monitor ligand binding in 2D spectra and for K_D determinations. X-ray crystallography was extensively used to determine the first structure depicting the PEX5-PTS1 interaction and to study the binding modes of multiple inhibitors. Many of the crystal structures determined in this work are good or excellent resolution and are analyzed very carefully. Impressively, Valeria Napolitano has not only identified a new lead compound to interfere with the PEX14-PEX5 interaction, but has also taken this as a starting point to synthesize an impressive array of analogues that require synthetic organic chemistry to make, and would be well out of the range of the average biochemist. The thesis even reports some "second generation" compounds, after pharmacokinetic studies showed that the initial lead compound had unfavorable metabolic properties.

Although this is perhaps the shortest PhD thesis that I have reviewed in a long time, it is among those with the most content. The presentation is extremely logical, and even Materials and Methods (often a tedious read) is informative, with a short summary of the basic principles underlying the method before the provision of detail. The only minor glitch in the presentation that I noticed is the "Errore, l'origine riferimento non è trovata" in quite a few places where a reference would be expected (e.g. p. 24). Perhaps this could be fixed before the thesis is made available online. Altogether, the work is the basis for four publications. At the time of submission of the thesis, two of these were already formally published (in J. Med. Chem. and Chem. Comm.), one was submitted to J. Med. Chem, and one was still in preparation. Impressively, Valeria Napolitano also lists no less than seven "other" publications in good journals, including another first author publication and second author publications, all published since 2016.

In summary: I am confident that this thesis **meets the requirements for a PhD degree and I also recommend a distinction.**

With best regards

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