IVYSPRING INTERNATIONAL PUBLISHER

Theranostics

2017; 7(11): 2965-2967. doi: 10.7150/thno.21603

Editorial

New Kid on the Block

Thomas Reinheckel ✉

Institute of Molecular Medicine and Cell Research, Faculty of Medicine, Albert-Ludwigs-University Freiburg, and German Cancer Consortium (DKTK), Partner site Freiburg, Freiburg, Germany

✉ Corresponding author: Tel: +49 761 203 9606. Email: thomas.reinheckel@mol-med.uni-freiburg.de

Received: 2017.06.30; Accepted: 2017.07.01; Published: 2017.07.30

Abstract

Cysteine cathepsins are a group of proteases involved in many physiological and pathological processes. Yet, the selective detection and inhibition of individual cathepsins is still challenging. This editorial is discussing the context of a recent work introducing a designed ankyrin repeat protein (DARPin) as novel approach for selective targeting of the protease cathepsin B.

Key words: Cathepsin B, DARPin, protease inhibition, protease imaging

In this issue of *Theranostics* a Slovenian-Swiss team of researchers presented their development of a DARPin (designed ankyrin repeat protein) for detection and inhibition of the protease cathepsin B.[1] I feel this elegant work opens a new avenue for cathepsin research and here I would like to argue for it.

The family of cysteine cathepsin proteases comprises 11 enzymes in humans and even 19 members in mice. The cathepsins are very potent proteases involved in intracellular nutrient homeostasis and extracellular tissue remodeling.[2] Cathepsins are also important for specialized pathways such as antigen presentation *via* the major histocompatibility complex II (MHC II). Likewise, some cathepsins are upregulated and involved in a variety of disease states such as kidney failure, atherosclerosis and cancer.[3,4] Much of the knowledge on cysteine cathepsins has been generated by scrutinizing genetically manipulated cell and animal models. Yet, this is hardly achievable in humans and hence, the selective detection, quantification and inhibition of those proteases in humans essentially requires a non-genetic toolset.

The development of those non-genetic tools represents a considerable and long-standing challenge. Why is this? The lead structure for cysteine cathepsins is that of the major protease of the papaya fruit – the papain. Consequently, all papain-like cysteine cathepsins share a very similar general fold and structure, and importantly, similar active sites. Although biochemical differences in substrate recognition and cleavage are known to the experts, one can see those proteases as potent enzymes with considerable promiscuity concerning their cleaved sequences, which is also dependent on the actual milieu such as pH conditions, redox state, and the extracellular matrix, such as presence of glucosaminoglycanes. It is thus hard to predict what cleavage is preferentially catalyzed in a physiological or pathological process by an individual cathepsin. In consequence, substrate-based probes for measuring individual cathepsin activities *in situ* or *in vivo* usually lack the selectivity suggested by *in vitro* studies in less complex systems. A major progress has been the development of the so-called activity based probes (APB).[5] Essentially, APB for cysteine cathepsins are enzyme inhibitors that covalently bind to the active site cysteine residue typical to all papain-like cysteine proteases. For measurement or visualization purpose, the APB is modified – in the more advanced versions of this approach by a quenched fluorophore. This fluorophore is de-quenched by proteolytic cleavage immediately prior to covalent inhibition of the enzyme, hence, this approach can be used to titrate the amount of active protease in a sample or

organism. Yet, also this approach bears unsolved caveats. One is the lack of sufficiently selective ABPs that discriminate the endoproteases of the enzyme family at low nano- or picomolar concentrations needed for robust *in vivo* approaches. The reason is that the "small molecule" ABPs provide relatively little opportunity for using the – often minimal - structural differences in or around the active site of the various cathepsins. Because of their versatility and high affinity of antigen binding, carefully selected monoclonal immunoglobulins could be very well suited for selective detection and also inhibition of cathepsins. Indeed, a monoclonal antibody inhibiting cathepsin B has been generated some years ago.[6] However, the antibody approach has not gained much momentum since then. Perhaps, the large size and complex architecture of immunoglobulins, the need for carful species adaptation, and the high expense for generating them make the antibodies a less attractive tool for extensive *in vivo* studies.

Now, Kramer & colleagues published their work in which they use another protein-based tool to detect and inhibit cathepsin B – a designed ankyrin repeat protein (DARPin). The DARPins are sometimes called "antibody mimetics", because they offer a similar versatility for interacting with their target proteins.[7] As DARPins are genetically engineered combinations of about five 3.5 kDa ankyrin repeat modules, they are only a fifth of the size of an immunoglobulin. Therefore, DARPins have higher tissue penetration and, because of the lack of Fc-domains used for recycling immunoglobulins by Fc Receptors, higher clearance rate than antibodies, which is important from the signal to noise aspect of imaging technology. To exploit those advantages of DARPins, the authors started with a DARPin library from which selected and characterized a DARPin as tool for *in vivo* inhibition and imaging of cathepsin B with a further potential to be used in drug delivery approaches. By structural analyses they could show the interaction of their best DARPin – called *8h6* – to cathepsin B. These analyses corroborated with the biochemical binding studies and enzyme inhibition kinetics. Impressively DARPin *8h6* binds the protease and inhibits its enzymatic activity at a concentration about five times below what is needed for cystatin C, which is the "best" endogenous cathepsin B inhibitor encoded in the human genome. Yet, *8h6* binds not only to the mature form of cathepsin B, but also to the cathepsin B zymogen in which substrate access to the active site is hindered by a pro-peptide. Interaction of the DARPin with the zymogen is less affine than the mature enzyme, however, during *in vivo* imaging studies it might be difficult to discriminate binding of *8h6* to the mature and zymogen forms and to use the approach as direct activity readout.

DARPin *8h6* might be of great help to solve another puzzle of cathepsin biology. Cathepsin B is highly abound inside cells where it is typically localized in the endolysosomal compartment. However, in cancer and other diseases cathepsins are also secreted into the tissue microenvironment. The smaller part of the secreted cathepsins are derived from the tumor cells, but the majority of extracellular cathepsins stems from tumor-associated immune cells with macrophages as the prominent example. In this situation it is a considerable experimental challenge to discriminate the homeostatic functions of the cathepsins inside cells from their extracellular effects that are thought to be tumor-promoting. From a therapeutic perspective it might be highly desirable to leave the intracellular homeostatic cathepsins intact, while inhibiting the extracellular proteases. The problem is that standard inhibitors or genetic approaches would target extra- and intracellular cathepsins to a similar extent, thereby preventing clear distinction of intra- and extracellular cathepsin functions. Kramer *et al.* demonstrated that DARPin *8h6* spares most normal tissues - except for liver - and accumulates predominantly in the stroma of the various mouse cancer models used. It seems that DARPin *8h6* is not efficiently taken up by the epithelial cancer cells, but much of the DARPin is phagocytosed by macrophages in the tumor stroma. However, it is fairly straightforward to minimize unwanted cellular uptake of 8h6, because a DARPin can be readily modified further by additional functional molecules. An example would be modification with poly(ethylene glycol) (PEG), which is well known for its ability to reduce cellular uptake. Using such tricks it might be possible to discriminate intra- and extracellular cathepsin functions in the near future. And of course, it is also possible to couple drugs onto the DARPin and to deliver them to the tumor site - an option that has not been explored in the current work.

In light of those exciting perspectives for the use of a cathepsin directed DARPin in theranostic approaches, the team around Prof. Boris Turk should be commended for their development of the first cathepsin selective DARPin. Welcome to the block!

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