The use of activity based protein profiling to study proteasome biology

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Chapter 4: Comparative Activity-Based Proteasome Profiling in Zebrafish and Mice

Introduction

In the last years, two different proteasome inhibitors (PIs) have been approved for the treatment of two hematopoietic cancers, multiple myeloma and mantle cell lymphoma [1]. Today proteasome inhibition is being investigated as a potential treatment for different diseases including solid tumors, muscle disorders, autoimmune syndromes and also in the field of organ transplants [2, 3]. Mammalian tissue can express up to six different catalytic proteasome subunits (up to seven in the thymus), which exhibit different substrate preferences. These subunits are basically assembled in two different proteasomes types, the constitutive proteasome, containing the β1, β2 and β5 subunits, and the immunoproteasome, where the constitutive subunits are replaced by their counter subunits β1i, β2i and β5i [4]. Bortezomib, the first PI approved in the clinic, shows selectivity towards the β5/5i and β1/1i subunits, being able to block them substantially while leaving the β2/2i activity almost unchanged. The second PI approved for the treatment of multiple myeloma is Carfilzomib, a β5/5i driven inhibitor, which at its therapeutic concentration partially also blocks the other proteasome subunits. The fact that both clinically accepted PIs show a subunit preference and that full proteasome inhibition is not necessary for its therapeutic benefit, has increased the effort of developing not only new and more potent inhibitors but also subunit specific inhibitors [5, 6]. The development of activity-based probes (ABPs) that facilitate the activity measurement of individual constitutive and immune subunits was one important step forward in the proteasome research field [7-9]. Broad-spectrum ABPs allow the simultaneous measurement of all different proteasome activities. Most of these pan-reactive ABPs do not show complete separation of the different proteasome subunits on SDS-PAGE, especially when immunoproteasomes are present. Due to the similar molecular weight of the β5/5i and the β1/1i subunits, resolving these subunits by SDS PAGE is complicated. Recently, several proteasome subunit specific inhibitors and probes have been developed, expanding the possibilities of inhibition using different combinations but also in a controlled manner, being possible to decide which subunit to inhibit and to what extent [10]. These subunit specific ABPs allow separation and direct activity determination of their target subunits. This has increased the knowledge about the different activities and substrate preferences of the proteasome active subunits, such as that more bulky, hydrophobic amino acids at P1 (first amino acid position after warhead) confer selectivity

towards immunoproteasomes, allowing the development of selective immunoproteasomes inhibitors [11, 12].

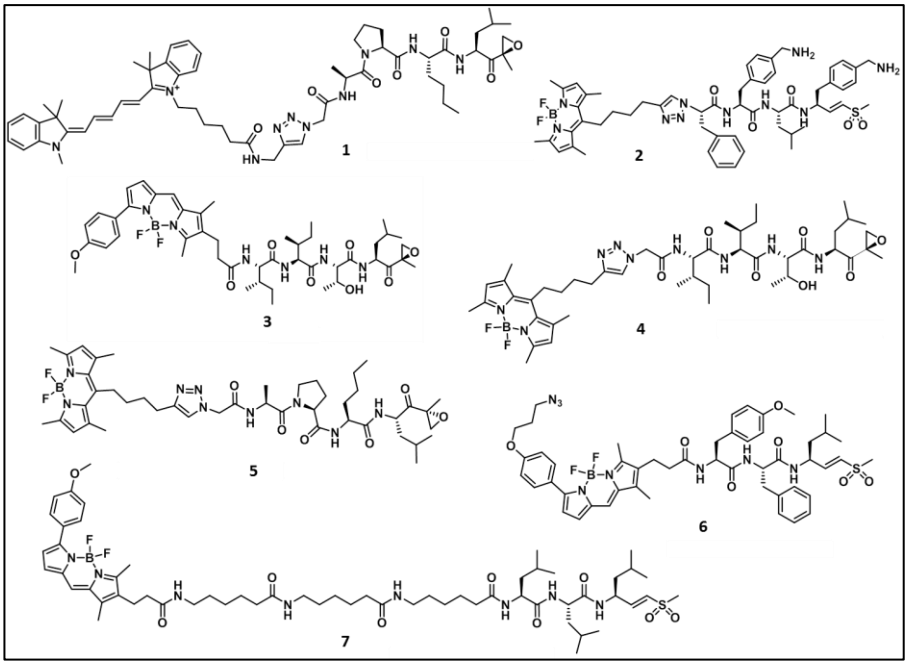

***Figure 1**. Structures of the activity-based probes used in this study. ABP **1**, Cy5-NC001, is β1/1i selective; ABP **2**, BODIPY(FL)-LU112, targets β2/2i; ABPs **3** (MVB003) and **4** (LWA300) are both an epoxomicin-based ABP with pan-reactive selectivity; probe **5** (LW124) is a β1/1i selective ABP; ABP **6**, BODIPY(TMR)-NC005, is a β5/5i targeting probe; ABP **7** (MV151) is a broad-spectrum proteasome probe.*

Most of the studies with PIs and ABPs have been mainly performed on human proteasomes and thus the newly synthetized inhibitors and probes have been chemically engineered to selectively target human proteasomes. Only a small fraction of these have been tested in other animals, mostly in mice. In this chapter, a pool of broad-spectrum and subunit selective ABPs (figure 1) has been screened in different murine organs and in zebrafish. Both organisms are broadly used in academic and clinic research, thus testing the applicability of these ABPs in these organisms will expand the usefulness of ABPP.

Results

Mice brain and testis, expressing only constitutive proteasomes, and spleen, which expresses both immuno- and constitutive proteasomes, were used in order to test the ABPs. Zebrafishes are only 1-3 cm and isolating their different organs is tidy and difficult. The easiest accessible organ suitable for extraction is the brain. Thus it was decided to perform the screen only on zebrafish brains and on full fish extracts. Figures 2 and 3 show a representative gel image for each ABP in the different murine and zebrafish tissues, and the optimal concentrations determined for each tissue are listed in table 1.

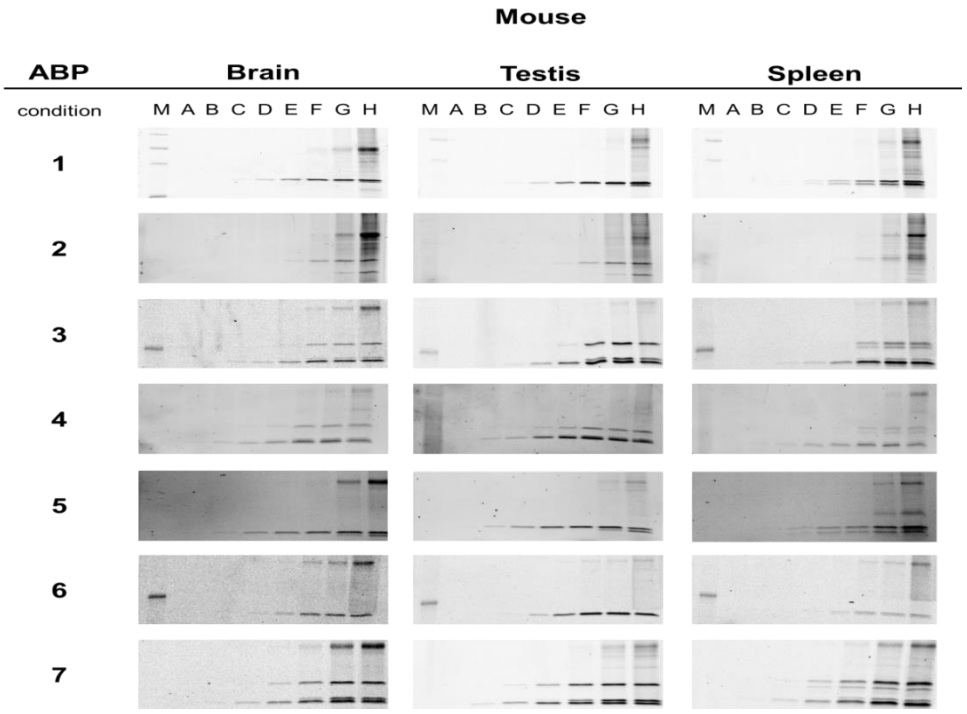

***Figure 2**. Representative SDS-PAGE gel images for each ABP in the mice tissues screened in this study. In the first lane of each gel the pre-stained protein marker was loaded (condition M). Highest concentration chosen for each probe was 10 µM (condition H). This concentration was diluted 5-fold in each lane ending with the end concentrations for each condition as follows: A: 0.13 nM; B: 0.64 nM; C: 3.2 nM; D: 16 nM; E: 80 nM; F: 400 nM; G: 2 µM and H: 10 µM.*

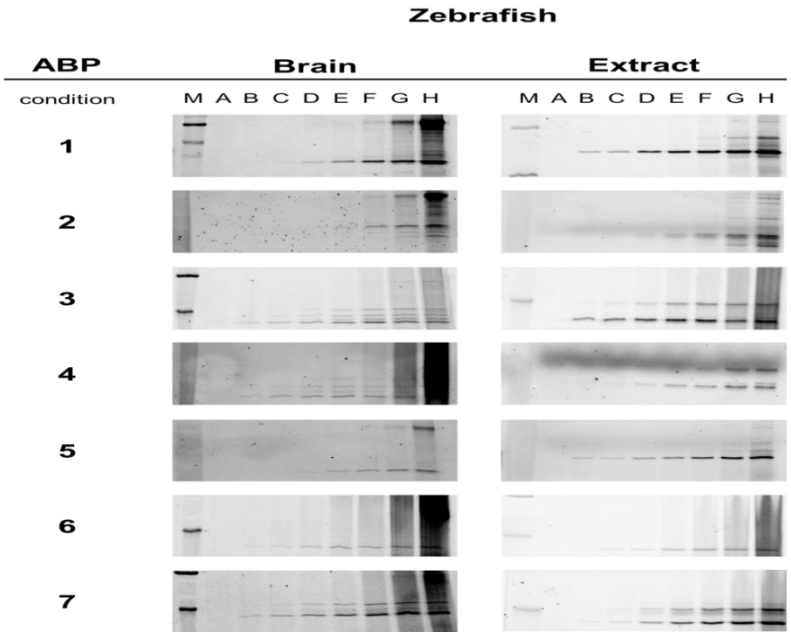

Figure 3. *Representative SDS-PAGE images for each ABP in the zebrafish. The ABPs concentrations where the following: A: 3 nM; B: 10 nM; C: 30 nM; D: 100 nM; E: 300 nM; F: 1 µM; G: 3 µM and H: 10 µM. M: protein marker.*

TISSUE	Activity-based Probe optimal concentration (µM)						
	1	2	3	4	5	6	7
Mouse Brain	0,4	0,4-2	0,4	0,4	0,1	0,4	2
Mouse Testis	0,4	0,4-2	0,4-2	0,4	0,1	0,4	2
Mouse Spleen	0,4-2	2	0,4-2	0,4-2	0,1-0,4	0,4	2
Zebrafish Brain	0,3-1	1	0,3-1	1	1	0,3-1	0,3-1
Zebrafish Extract	0,3-1	1	0,3-1	3	1	0,3-1	1-3
Human cell line extracts	0,1	0,25	0,25-0,5	0,25-0,5	0,1	0,25	0,5-1

Table 1*. Optimal labeling concentrations for each ABP in the different tissues and organisms.*

All probes retain their selectivity towards the proteasome subunits while their optimal labeling concentrations are, in general, slightly higher than the ones used in human cell lines extracts. β1/1i selective probes **1** and **5** and pan-reactive ABPs **3** and **7** show a similar pattern in all different tissues as the one observed in human cell line extracts, a high selectivity towards the proteasome and off-targets are only visible at high concentrations.

Interestingly, ABP **4**, a Cy2-fluorescent analog of probe **3**, shows lower overall signal/noise ratios compared to ABP **3**. This is especially significant in the murine spleen and in the zebrafish samples, where the β2/2i bands are hardly visible due to low band intensity (figure 2) or high background (figure 3). This high background labeling at high probe concentrations in zebrafish brain and extracts is in general obtained with most of the probes. ABPs **2** and **6** label efficiently the β2 and β5 subunits, respectively, in all screened tissues but murine spleen. For both probes the bands are very weak compared to their performance in the other tissues. In the case of ABP **2** the concentrations needed to obtain labeling are much higher than the ones used for human proteasome labeling and in some cases, like in the spleen (figure 2), at the optimal labeling concentration shows some off-target bands. Interestingly, although ABP **6** labels β5 or β5i selectively, the separation of these two subunits was poorly achieved on SDS-PAGE (figure 2, spleen), while they were proven to separate properly human β5/5i subunits. To check whether this was due to poor subunit separation or to the ABP being only able to label one of the subunits, a 2D-gel electrophoresis was performed. Three distinct stripes can be observed in the middle of the 2D-gel (figure 4), two having a bright signal and the third a much lighter one. This third stripe seems to run lower than the other two, suggesting that this might be the stripe for the labeled β5i subunit and the brighter stripes might correspond to the β5 subunits. Their position in the gel compared to the marker and their separation is similar to the one observed for human subunits (figure 4).

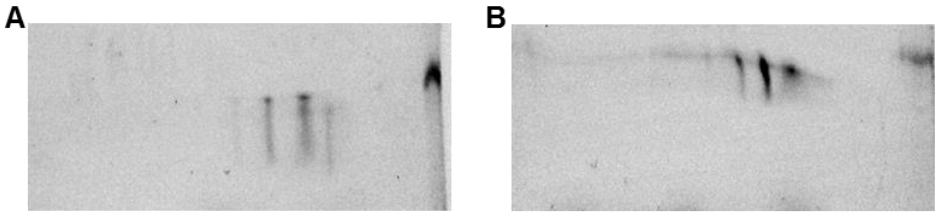

***Figure 4**. 2-D SDS-gel image of a mouse cell lysate (A) and a human cell line extract (B), which were incubated with ABP **6** at the concentration of 0.5 μM for 1 hour. Sample were loaded on a non-linear pH gradient (3-10) strip and resolved on 12.5% SDS-gel after isoelectric focusing. A pre-stained marker was added to the SDS-gel (bright band on the right side of the image). As it can be seen in both gel images, two isoforms of the β5c subunit are visible (the two left stripes). The β5i subunit can be visualized running a slightly lower on the gel than the β5c subunit bands.*

Discussion

All here tested ABPs target selectively the proteasome subunits from both organisms. Although losing a bit of potency compared to their performance in human cell lines extracts, this difference is very small and all probes maintain a low micromolar concentration for optimal labeling (table 1). For some probes, the concentrations needed in the murine spleen are slightly higher than the ones in the other murine tissues. This can possibly be explained by the fact that this organ expresses both immuno- and constitutive proteasomes, and is probable that the total amount of proteasomes is higher than in the other tissues where only one type of proteasomes is expressed. Table 2 shows the alignment percentage of the mouse and zebrafish subunits with the human ones. The largest differences with the human amino acid sequence are found in zebrafish, especially for the β1i and the β2i subunits. These differences might explain the probe potency variation when used in zebrafish, and the poor resolution found when labeling these subunits with the selective β1-directed ABP **1** or the β2-directed ABP **2.**

		β5	**β5i**	**β1**	**β1i**	**β2**	**β2i**
Mouse	identity (%)	95	93	97	90	97	89
	similarity (%)	97	98	98	98	99	93
Zebrafish	identity (%)	84	80	79	67	82	56
	similarity (%)	91	91	92	84	94	72

Table 2*. Alignment percentage of the proteasome active subunits amino acid sequence from mouse and zebrafish with the human subunits. Identity is showing the percentage of shared amino acids; similarity includes the amino acid replacement by another with similar characteristics.*

As can be seen in figure 1, ABPs **3** and **4** have the same peptide backbone and only differ in their fluorescent tag, BODIPY(TMR) and BODIPY(FL) respectively. In the case of human or mice proteasomes this does not influence their output, both sharing a similar optimal concentration, although the signal obtained with probe **4** in spleen is lower than in the other tissues. In zebrafish this difference increases up to three times. This increase seems to be due to the reporting tag of **4**. It causes a large background signal, making it hard to properly visualize the proteasome bands. This phenomenon of high background noise is especially significant for **4** but not for the other ABPs with a BODIPY(FL) fluorescent tag, probes **5** and **2**. This indicates that is not due to the reporting group but more likely a

combination of fluorophore and the inhibitor backbone. Although the three BODIPY(FL)-bearing ABPs have an acceptable signal to noise ratio in zebrafish, when labeling full fish extracts a vague and diffuse band shows around 35 kDa (figure 3). This band is probably an endogenous fluorescent protein that can interfere specially with the identification or quantification of the β2 subunits due to their close position in the gel. A straightforward strategy to avoid this fluorescent protein interfering with the band analysis could be to use an ABP with a different fluorescent tag, scanning the gel in a different fluorescent channel. Another possibility is to precipitate the proteins, for example by a chloroform/methanol precipitation, which might remove the fluorescent molecules. Subjecting the sample after ABP exposure to a size-exclusion column might separate all small proteins from the large protein complexes like proteasomes, which has been successfully proven in the Chapter 3 of this thesis, since ABPs label only active subunits of fully assembled proteasomes but not single subunits.

Another interesting feature in the zebrafish brains and full extracts is the additional band or smear that appear between the β2 and the β1/5 subunits when incubating them with high concentrations of ABPs **2** or **7** (figure 3). These could be off-targets of the probes, but since they are only visible in zebrafish and not on mice or human samples it should be a unique zebrafish protein. Another possibility is that these probes also label post-translationally modified β2 subunits. This theory is supported by the fact that ABP **2** does not show a sharp β2/2i band, as it does for mouse, but a wide and diffuse one. This is in concordance with PTMs (post-translational modifications), as depending on the type and the amount they may influence and vary the molecular weight and charge of the modified protein thus shifting its position in the gel. These diffuse or extra bands are only visible with these two ABPs but not with the other probes, which also label the β2/2i subunits, ABPs **4** and **5**. The main difference between these probes and the rest are the warheads, ABPs **2** and **7** have a vinyl sulfone warhead while probes **4** and **5** have an epoxyketone. It seems that vinyl sulfone probes are the only ones that label and separate these modified subunits. Further experiments, like on-gel digest or pull-downs need to be performed in order to validate this hypothesis and identify these extra bands.

Off-targets or high background labeling is only observed when incubating ABPs at high concentrations. The high background labeling is mainly observed in zebrafish brains and also in extracts but it is not obtained in the murine tissues, suggesting that the probe off-targets could be larger in zebrafish than in mice. Another explanation for this high background labeling could be that the signal to noise ratio is not very large, thus when adjusting the image contrast to obtain substantial signal, the background at high probe concentration gets also larger due to the ABPs probably just sticking to proteins. Washing away the excess of probe after the incubation period might be beneficial to remove the

high background due to stickiness of the ABPs and will show how much of this background is actual an off-target of the probe, which could be then easily identified by in-gel digestion coupled to mass analysis.

Conclusion

In conclusion, all here used ABPs are suitable for their application in mouse and zebrafish, although the probes show less potency in mouse and zebrafish in comparison to human proteasome labeling. The largest difference is found in zebrafishes where the optimal concentrations are in some cases three times higher than in human and where optimal labeling from the immunoproteasome subunits β1i and β2i is compromised (figure 2). The low alignment percentage of these subunits between human and zebrafish (table 2) might explain the low efficiency of the probes labeling these subunits. This leaves room for improvement in generating ABPs that can bind to many species with a comparable potency and selectivity, but also in the production of an organism-selective proteasome inhibitor or probe. If an organism-selective inhibitor could be developed, it might be a plausible treatment against infections for example, thus allowing the targeting of only non-host cells, but it could also be beneficial for the food industry plague control by using human harmless chemicals to fight the responsible organisms that cause the plague.

Experimental procedures

Animals and tissues

Mouse organs were isolated from adult mice. Zebrafish brains were isolated from adult zebrafish while full body extracts were obtained from zebrafish larvae.

Activity-based protein profiling

Organ tissues were homogenized in 3 volumes of lysis buffer (50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.025% digitonin and 0.2% NP40) with a tissue homogenizer and further disrupted by 30 seconds sonication. After cold centrifugation at 13.000 rpm for 10 min, protein concentration was measured with the Qubit Protein Assay on the soluble fraction and kept at -80 ºC until use. Zebrafish full body extract supernatants were cold centrifuged again at 30.000 *g* for 60 min to separate the membrane fraction and to pellet cell debris. The protein concentration in the soluble fraction was measured as before and stored at -80ºC. Equal amounts of protein were incubated with different concentrations of ABP for 1 h at 37ºC, resolved by 12.5% SDS-PAGE and scanned with the ChemiDoc™ MP System with the Cy2, Cy3 and Cy5 settings. Commassie

blue staining was used as loading control. All gel images were analyzed by the Image Lab software (Bio-Rad).

2D-gel electrophoresis

Before starting the protocol note that the fluorescent probes are light sensitive and every 1-3 hour it will lose half of fluorescent intensity in the final result. The samples were kept in the dark or covered with aluminium foil as much as possible.

Some 100 µg protein was taken in 90µL total volume with lysis buffer and 10 µL (10xstock) of probe was added. Sample was incubated for 1 h at 37 ^{o}C prior precipitation with TCA by adding 25 µL of 70% TCA and incubating for 0.5 h on ice (Should see the liquid becoming cloudy as the protein precipitates out). After cold centrifugation for 5 min at 14000 rpm the supernatant was removed and the pellet washed twice with 500 µL ice-cold acetone (if the pellet comes loose during washing repeat centrifugation step with acetone). Sample was dried out in a speed vac overnight. Pellet can be stored at -20 ^{o}C until use. Pellet was solved in 150 µL Urea buffer (30 mM Tris-HCl pH 7.5, 7.7 M urea, 2.2 M thiourea and 4% CHAPS) with 3uL Destreak agent (end concentration 0.5%) and 0.75uL IPG buffer (3-10) (end concentration 2%) was added freshly to the solution (may take very long to dissolve; to speed up the process the solution can be warmed up to 37 ^{o}C, vortexed, or sonicated). A portion of the solution can be stored to run on normal 12.5% SDS gel if desired. The lane from the incubation cassette was filled from the non-tilted edge with the sample. Slowly the strip was put onto the lysate (gel side down and make sure there are no bubbles under the strip). 2ml of mineral oil was loaded over the top of it to prevent the solution from evaporating. It was incubated between 24 and 96 h. After rinsing the strip with distilled water it was loaded into the focusing basket with the correct orientation and wet Whatman paper was used to separate the strip form the wires. The lane was again covered with mineral oil. The following focusing program was used: Step 1, 0.1 min 50 V; Step 2, 30 min 200 V; Step 3, 30 min 200 V; Step 4 30 min 400 V; Step 5, 30 min 400 V; Step 6, 30 min 600 V; Step 7, 30 min 600 V; Step 8, 60 min 3500 V; Step 9, 240 min 3500 V; Step 10, 10 min 200 V; Step 11, up to x hours 200 V. Afterwards the strip was incubated in 2 mL equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 20% SDS and bromo phenol blue) with 10 mg/mL DTT for 1 h at room temperature prior 1 h incubation with 2mL equilibration buffer with 25 mg/ml iodoacetamide. The strip was loaded on a 12.5% SDS-PAGE gel only consisting of running gel with the help of a 1% agarose solution to prevent air bubbles between the strip and the gel. (NOTE: Load the strip quickly, and in a diagonal fashion starting with one corner of the strip and then easing the rest of the strip in to allow air bubbles to escape. The agarose solution hardens quickly, if the loading failed, remove the agarose and try again. A slot in the agarose can be made to be filled up with protein marker). The gel was ran at 300 V for 60-75 min (Encase the running container in an ice bath to prevent excess of temperature in the gel)

References

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