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Title: Activity-based proteasome profiling
Issue Date: 2013-12-16

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Activity-based protein profiling:

an enabling technology in chemical biology research

2.1 Introduction

Amongst the toolkit of functional proteomic techniques, ABPP has proved powerful and attractive for its remarkable ability to label and enrich variable enzymatic activities. Activity-based probes (ABPs) can be viewed as chemical antibodies to report on the expression of a protein, but at the same time as probes to detect the target active enzymes in a living system (see Figure 1 for a general representation of the workflow). ABPP has been used on protein extracts, on living cells, and sometimes even on animal models. In this review some highlights of ABPP in chemical biological research during the past two years will be discussed, with a specific focus on applications in the fields of biochemistry, molecular and cellular biology, medicinal chemistry, pathology, physiology and pharmacology. The structures of the activity-based probes discussed here are compiled in Figure 2 and cross-referenced in bold numbers in the text.

2.2 ABPP in biochemistry

Since the invention of the methodology, ABPP has proven highly useful for the identification and annotation of enzymatic activities and their role in important biochemical pathways. One research area that has benefited in particular from ABPP is that involving the ubiquitin-proteasome system. For instance, recently a thymus specific proteasome was identified in which the β5t subunit replaces β5i in the immunoproteasome to create a new particle called the thymoproteasome(*1*). Affinity purification of the active site fragment coupled to biotin-epoxomicin followed by LC/MS protein identification demonstrated that β5t is catalytically active in murine thymus, and an ensuing ABPP-based competition assay employing proteasome ABPs **1** and **2** pointed towards a preference of β5t for neutral, hydrophilic substrates, in contrast to the hydrophobic substrates preferred by β5/β5i(*2*). In a second example of ABPP application to the UPS system, C-terminally modified, HA-tagged ubiquitin derivatives were applied as activity-based probe (**3**) for the profiling of a whole range of deubiquitinating enzymes (DUBs) and ubiquitin ligases,

demonstrating their catalytic activity and delivering the research tools for probing their involvement in protein ubiquitilation events(3).
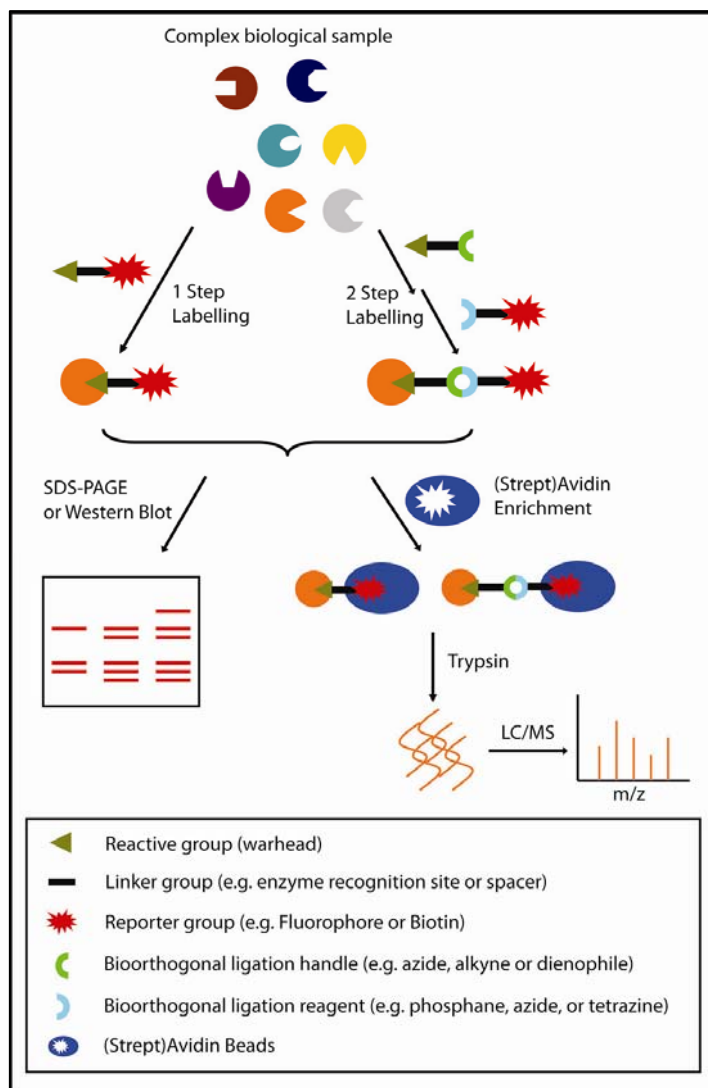
Figure 1: General scheme of activity-based protein profiling experiments. Complex proteomes are exposed to a type of activity-based probe either in vitro or in situ and the labeled protein targets are affinity-purified, separated, visualized and identified with a proteomics analysis system of choice: SDS-PAGE, western blotting or liquid chromatography hyphenated to mass spectrometry (LC-MS). A two-step labeling strategy is optional in case that the reporter tag obstructs the cell permeability of the ABP or the interaction between the ABP and target protein.

In multiple human diseases such as rheumatoid arthritis, cancer and colitis the enzymatic activity of protein arginine deiminase 4 (PAD4), which catalyzes the hydrolysis of peptidyl-arginine to peptidyl-citrulline was found to be dysfunctional, possibly due to autodeimination. In order to test this hypothesis, a PAD4 selective probe (**4**) was used to affinity purify PAD4 from living cells together with several binding partners including histone H3, the histone deacetylase HDAC1 and p53(4). It was shown that PAD4 autodeimination does not alter its activity, substrate specificity, or calcium dependence. Autodeimination however modulates the ability of PAD4 to interact with its previously identified binding partners(5). The same researchers investigated the regulation of PRMT1 protein arginine methyltransferase 1 (PRMT1). In the presence of estrogen, PRMT1 methylates Arg260 of the estrogen receptor, triggering the activation of protein kinase B (PKB/Akt) and promoting cell survival. A substrate-based ABP (**5**) showed that the PRMT1

activity is regulated, both temporally and spatially, in response to estrogen(*6*).

ABPP can be combined with LC/MS-based protein quantification platforms, allowing for accurate quantification of enzymatic activities. In a recent and highly innovative application, iodoacetamide (IA) alkyne (**6**) was used to determine the global reactivity profile of cysteine thiols across the entire human proteome(*7*). Substoichiometric amounts of the probe relative to the total number of cysteines were applied to modify the most reactive, and hence functionally involved (as for instance in enzyme active sites) cysteine thiols. In a comparative experiment extracts from the same biological source were saturated with **6** to modify all cysteines. The pool of hyper-reactive cysteines was click-ligated to a heavy N_3-stable isotopic-TEV tag and the comparative pool to the light form of the tag. Both pools were combined and ensuing enzymatic digestion, affinity purification and LC/MS analysis of peptides resulted in a global map of cysteine reactivity that is instrumental for the prediction and annotation of functional cysteines. In another example, Cravatt and co-workers combined stable isotopic labeling in cell culture (SILAC) with their Fluorophosphonate (FP) ABPP methodology to quantify the inhibition of serine hydrolyses(*8*). The ABP-enriched enzymes from samples with or without inhibitor (containing differentially labeled lysines and arginines) were mixed and analyzed by LC/MS. By comparing the intensity of the assigned peaks of the target enzyme, enzyme inhibition was quantified.

2.3 ABPP in molecular and cellular biology

Visualizing active proteins in living systems requires a robust signal to noise ratio involving fluorescent labels that emit at higher wavelengths compared to the biologic auto-fluorescence background of around 480-500 nm. Cysteine dependent cathepsins function mainly in the endo-lysosomal compartments catalyzing the hydrolysis of intra- and extra-cellular proteins but are also associated with tumor formation, growth, invasiveness and metastasis. Cathepsins B and L in particular are highly expressed in various tumors and are thus promising targets for tumor diagnosis and monitoring of therapy. The Bogyo lab developed a fluorescently quenched cathepsin probe (**7**) for the noninvasive optical imaging of subcutaneously grafted tumors in mice(*9*). Upon intravenous (i.v.) administration, the quencher is cleaved by active cathepsins and a sharp increase in fluorescence signal of the near infrared fluorophore Cy5 is detected in and around the tumors. A second example is the development of a potent and selective ABP (**8**) bearing a near infrared fluorophore for in vivo imaging of legumain(*10*). Legumain is a lysosomal protease involved in antigen processing and matrix degradation, but is also upregulated during tumorigenesis. The ABP enabled monitoring legumain activity in normal tissues, in solid tumors by high contrast shortly after i.v administration and the tracking of whole body distribution of the probe as well as the level of active legumain in organs by ex vivo imaging and SDS-PAGE.

In macrophages and dendritic cells, elevated cathepsin activities are required for antigen processing and presentation. In order to target and monitor cysteine cathepsins in professional antigen presenting cells, a mannose cluster was clicked to the Bodipy-TMR (tetramethylrhodamine) equipped cathepsin probe DCG04 (**9**). Probe **9** was taken up by specific receptor mediated transport via the mannose receptor and selectively labeled active cathepsins in cell culture(*11*).

Caspase activities are early mediators of apoptosis. Imaging and quantification of caspase activities holds promise for early diagnosis or disease monitoring, for instance after exposure to anti-tumor drugs in clinic. A cell permeable, caspase 3 and 7 sensitive ABP (**10**) equipped with a near-infrared fluorophore revealed dexamethasone-induced apoptosis in murine thymi and in tumor grafted mice treated with the apoptosis-inducing monoclonal antibody Apomab(*12*). Maximum fluorescence signal in live mice coincided with peak caspase activity assessed by SDS-PAGE analysis, emphasizing the potential of the probes for in vivo non-invasive optical imaging in preclinical and perhaps clinical settings.

Recently, quinone methide chemistry was utilized to develop a series of scaffolds in a highly modular fashion and with facile interchangeability of the moieties for making a series of caspase and phosphatase activity-based probes (**11**). Several active caspases were labeled both in vitro and in digitonin permeated cells and the quenched, two photon activatable fluorescent labels allowed sensitive detection of the target enzymes(*13*).

ABPs specific for the proteasome were instrumental in providing evidence for a remarkable discovery in the field of immune biology(*14*). Lymphocytes involved in an immune response undergo vigorous cell division to amplify their numbers and small fluctuations in the critical T-box transcription factor (T-bet) severely impacts on T-cell progeny where naïve CD8+ T-cells differentiate towards effector but not memory fate, while CD4+ T-cells developed towards T helper 1 (Th1) and less Th2 or Th17 cells. Flow cytometry and fluorescence microscopy using proteasome ABPs **1**, **2** and **12** showed that T-bet levels are under proteasomal control and that during mitosis, proteasome activity is asymmetrically distributed between daughter cells as shown in Figure 3. Unequal proteasome distribution determines T-cell lineage fate and thus the direction of immune response development.

2.4 ABPP in medicinal chemistry

Both the FDA approved antiobesity drug orlistat, potentially an antitumor agent, and the nucleoside antibiotic showdomycin are covalently binding to their cellular targets(*15, 16*). For target identification, an alkyne group was introduced to minimally change the chemical structure and that enabled post-lysis bio-orthogonal ligation of reporter or affinity tags followed by either fluorescent imaging or LC/MS based protein identification (**13, 14**). Orlistat showed 8 off-targets next to the known thioesterase domain

of fatty acid synthase (FAS). The antibiotic effect of showdomycin against Staphylococcus aureus in turn might involve inhibition of the essential enzymes MurA1 and MurA2 that are required for cell wall biosynthesis.
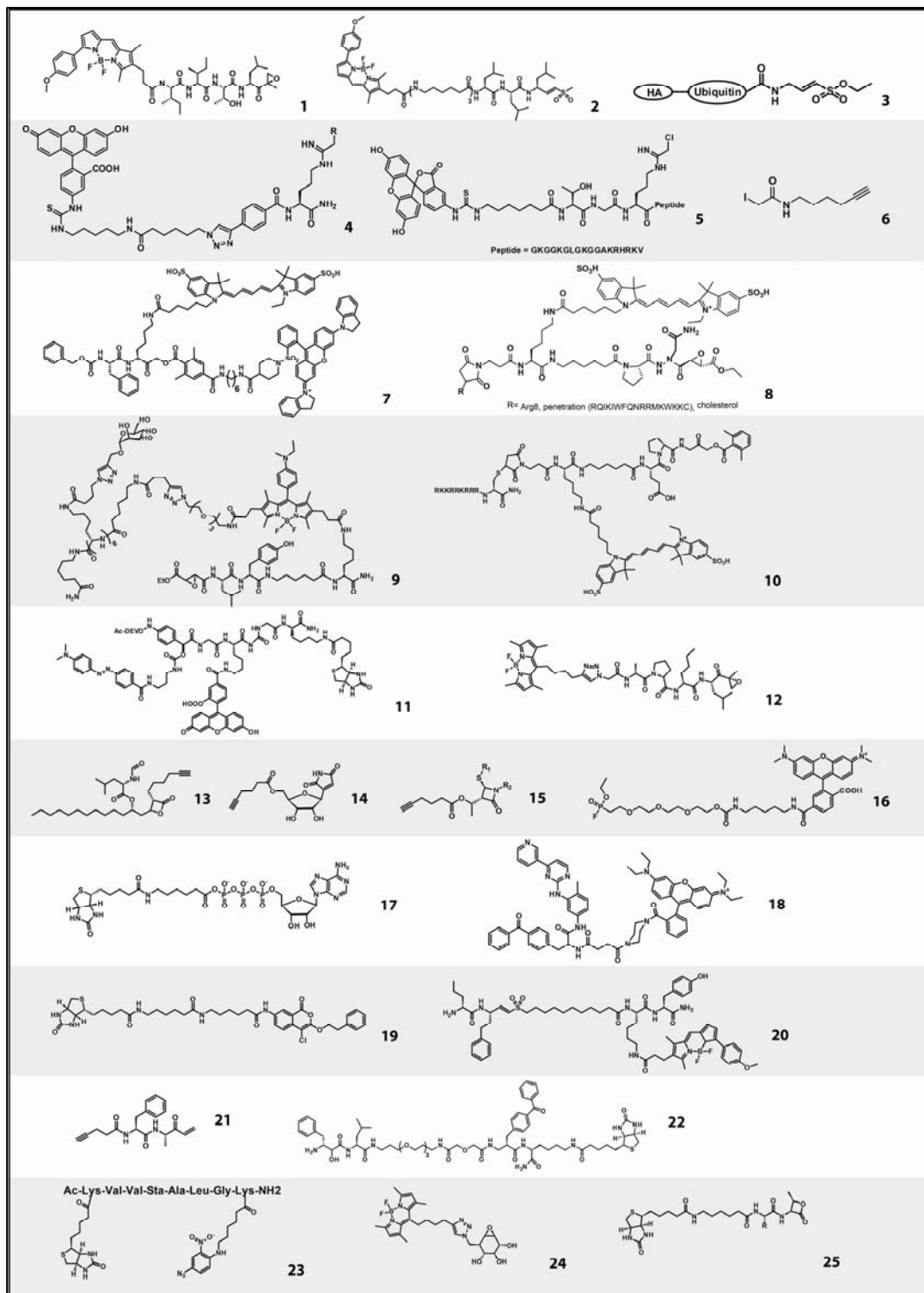
Figure 2: Chemical structure of Activity-Based Probes: 1 reference (2, 14); 2 reference (2, 14, 24); 3 reference (3); 4 reference (4,5); 5 reference (6); 6 reference (7); 7 reference (9); 8

reference (10); 9 reference (11); 10 reference (12); 11 reference (13); 12 reference (14); 13 reference (15); 14 reference (18); 15 reference (18); 16 reference (19, 23, 29, 31); 17 reference (20, 21); 18 reference (22); 19 reference (25); 20 reference (25); 21 reference (26); 22 reference (27); 23 reference (28); 24 reference (30); 25 reference (32).

The non-covalent antibiotic vancomycin was equipped by the Sieber lab with an alkyne group and a photo-activatable crosslinker(*17*). As expected, the vancomycin probe binds to the D-Ala-D-Ala motif of nascent peptidoglycan disrupting cell wall biosynthesis followed by autolysin (Atl) triggered cell rupture and death. Surprisingly, the probe also binds and inhibits the Atl amidase domain that causes massive defects in cell morphology and enhances the tolerance of S. aureus to low concentrations of vancomycin.

Not only the drug target proteins but also the resistance related proteins can be identified by ABPP. Small synthetic β-lactam probes (**15**) were applied to comparatively profile in situ enzyme activities of wild type and methicilin resistant S. aureus (MRSA) strains revealed unique MRSA features as known resistant associated targets, involved in cell wall biosynthesis and antibiotic sensing, but also uncharacterized enzymes capable of hydrolyzing β-lactam moieties(*18*). Such tools might prove their value for the identification of resistance genes and help to discover new drug targets for customized therapeutic interventions.

The design, synthesis and screening of compound libraries are important activities in medicinal chemistry and ABPP comes in useful when screening for enzyme inhibitors is the subject of study. A high-throughput screening protocol based on the FP-rhodamine probe (**16**) was developed to identify selective and potent inhibitors for two unrelated and poorly characterized serine hydrolases, namely retinoblastoma-binding protein-9 (RBBP9) and thioltransferase glutathione S-transferase omega 1 (GSTO1) both suggested to be cancer related genes(*19*). The enzymes were incubated with a compound library in a 384 well format, followed by FP-rhodamine labeling of residual enzyme activity. The bioactive alkaloid emetine was identified as a selective inhibitor of RBBP9. GSTO1 was found to be a target of several electrophilic compounds (including omeprazole and rifampicin) present in public libraries.

The KiNativ high-throughput screening platform from ActivX employs an ATP-analogue probe (**17**) for capturing ATP processing enzymes and profiling several well studied kinase inhibitors against >200 kinases in native cell proteomes to reveal biological targets for some of the inhibitors(*20, 21*). The authors found several striking differences between native and recombinant kinase inhibitory profiles, in particular, for the Raf kinases. This highlights the complexities of protein kinase behavior in the cellular context and demonstrates that profiling results based on recombinant/purified enzymes can be misleading. An Abelson (Abl) tyrosine kinase, the molecular target linked to the

development of chronic myelogenous leukemia (CML), specific ABP (**18**) was synthesized inspired by the clinically used Imatinib drug and equipped with a photo-activatable crosslinker(*22*). These examples indicate the power of ABPP for screening and development of new therapeutic strategies.
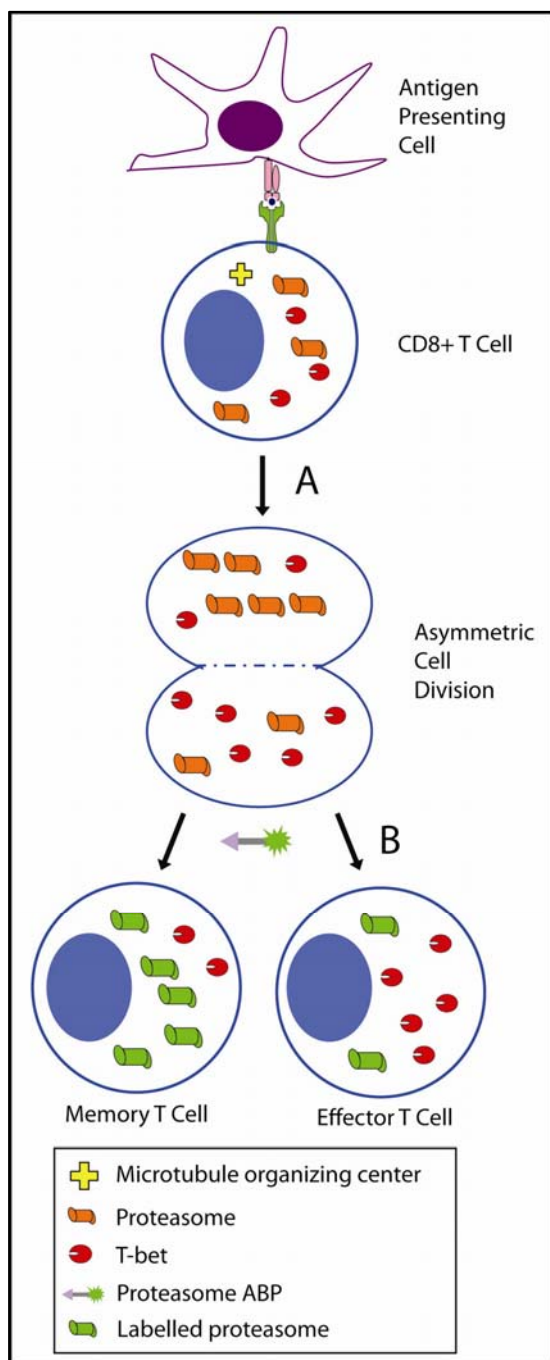
Figure 3: Asymmetric T-cell division revealed by activity-based protein profiling of the proteasome activity (*14*). Upon stimulation by professional antigen presenting cells, CD8+ and CD4+ T-cells can undergo asymmetric cell division producing daughters of different types. ABPP assays showed that asymmetric proteasome segregation during mitosis dictates the T-bet transcription factor concentration that changes the fate of the daughter cells.

2.5 ABPP in pathology, physiology and pharmacology

Since ABPP is a powerful tool to observe the activity of a wide range of functional enzymes, it can be used as a sensitive method for diagnosis and prognosis of a series of diseases, which are related to some abnormal enzymatic activities, such as cancer,

pathogen infection, and metabolic disorders. Tumor cells display progressive changes in metabolism that correlate with malignancy, including development of a lipogenic phenotype. The Cravatt lab measured the activity of a series of monoacylglycerol lipases (MAGL) with ABPP (**16**) in both aggressive and nonaggressive human cancer cell lines(*23*). Elevated MAGL activity was found in the aggressive cancer lines and primary tumors where MAGL regulates a fatty acid network enriched in oncogenic signaling lipids that promotes migration, invasion, survival, and in vivo tumor growth. An example of ABPP in studying tumor pharmacology is the use of MV151 (**2**) for profiling the proteasome activity of bortezomib sensitive and resistant multiple myeloma (MM) cells(*24*). Multiple myeloma is an aggressive malignance of plasma B-cells, which can be treated with bortezomib, a proteasome inhibitor that blocks the $\beta 5$ and $\beta 1$ subunits. Driessen and co-workers found that elevated transcription rates, activities and polypeptide levels of $\beta 5$, $\beta 1$ and $\beta 2$ subunits combined with increased expression and proteasome association of the 11S proteasome activator were the main pathways for resistant MM cells to cope with Bortezomib stress.

The ABPP approach was used in the Bogyo lab to identify essential proteases required for the proliferation of parasites. ABPs **19** and **20** were used to identify the malaria proteases pfSUB1 and DPAP3 as the key regulators of erythrocyte rupture(*25*). Recently, the same group identified the parasite protease TgDJ-1 in Toxoplasma gondii (**21**), which plays a key role in the pathogen attachment and invasion of host cells(*26*). Harbut and colleagues used bestatin analogues (**22**) to identify the malaria parasite aminopeptidases pfA-M1 and pf-LAP, which are necessary peptidases for hemoglobin digestion and parasite early life cycle(*27*). Various peptidase activities were profiled by ABPP (**23**) in tick GI-tract, which are often carriers of parasites. The study revealed the way hemoglobin from human blood was digested in tick GI-tract by a multipeptidase pathway(*28*). Hepatitis c virus (HCV) infection is a global harmful disease with unclear pathogenesis. Pezacki and co-workers used FP-Rhodamine (**16**) to visualize the differential host enzyme activation during the HCV replication and identified carboxylesterase 1 to play an important role in HCV propagation(*29*).

Gaucher disease, a common lysosomal storage disorder, is often underlined by the deficiency of glucocerebrosidase (GBA). Coupling the GBA inhibitor cyclophellitol to different Bodipy fluorescent groups provided ABPs (**24**) of ultra-high sensitivity and specificity for GBA(*30*). The probes were tested and validated both in vitro and in vivo and will find application in screens for new GBA inhibitors or chemical chaperones in living cells and for the diagnosis or therapy progression in Gaucher disease by quantifying the GBA activity in patient materials.

ABPP has also been used to study plant pathology and physiologic problems. FP probes (**16**) were used to visualize the differential activation of serine hydrolases in the unchallenged and botrytis-infected Arabidopsis thaliana(*31*). In another work, beta-lactone

probes (**25**) were used to identify a papain-like peptide ligase in the same organism(*32*).

2.6 Conclusion and outlook

Thanks to the discovery of new warheads, the design of improved enzyme targeting moieties and reporter/affinity tags, in conjunction with the remarkable increase in sensitivity, resolution and dynamic range of detection instruments, ABPP has become a powerful functional proteomics tool in the field of the biochemistry, (molecular) cell biology, medicinal chemistry, physiology, pathology and pharmacology. However, to date many enzyme families defy modification by ABPs and future research will reveal the extent by which ABPP can be applied to different enzyme families and indeed also non-enzymatic protein families.

References and notes

1.   Murata, S., Sasaki, K., Kishimoto, T., Niwa, S., Hayashi, H., Takahama, Y., and Tanaka, K. (2007) Regulation of CD8+ T cell development by thymus-specific proteasomes, *Science 316*, 1349-1353.

2.   Florea, B. I., Verdoes, M., Li, N., van der Linden, W. A., Geurink, P. P., van den Elst, H., Hofmann, T., de Ru, A., van Veelen, P. A., Tanaka, K., Sasaki, K., Murata, S., den Dulk, H., Brouwer, J., Ossendorp, F. A., Kisselev, A. F., and Overkleeft, H. S. (2010) Activity-Based Profiling Reveals Reactivity of the Murine Thymoproteasome-Specific Subunit beta 5t, *Chem Biol 17*, 795-801.

3.   Love, K. R., Pandya, R. K., Spooner, E., and Ploegh, H. L. (2009) Ubiquitin C-Terminal Electrophiles Are Activity-Based Probes for Identification and Mechanistic Study of Ubiquitin Conjugating Machinery, *Acs Chem Biol 4*, 275-287.

4.   Slack, J. L., Causey, C. P., Luo, Y., and Thompson, P. R. (2011) Development and Use of Clickable Activity Based Protein Profiling Agents for Protein Arginine Deiminase 4, *Acs Chem Biol 6*, 466-476.

5.   Slack, J. L., Jones, L. E., Bhatia, M. M., and Thompson, P. R. (2011) Autodeimination of Protein Arginine Deiminase 4 Alters Protein-Protein Interactions but Not Activity, *Biochemistry-Us 50*, 3997-4010.

6.   Obianyo, O., Causey, C. P., Jones, J. E., and Thompson, P. R. (2011) Activity-Based Protein Profiling of Protein Arginine Methyltransferase 1, *Acs Chem Biol 6*, 1127-1135.

7.   Weerapana, E., Wang, C., Simon, G. M., Richter, F., Khare, S., Dillon, M. B. D., Bachovchin, D. A., Mowen, K., Baker, D., and Cravatt, B. F. (2010) Quantitative reactivity profiling predicts functional cysteines in proteomes, *Nature 468*, 790-U779.

8.   Adibekian, A., Martin, B. R., Wang, C., Hsu, K. L., Bachovchin, D. A., Niessen, S., Hoover, H., and Cravatt, B. F. (2011) Click-generated triazole ureas as ultrapotent in vivo-active serine hydrolase inhibitors, *Nat Chem Biol 7*, 469-478.

9.   Blum, G., von Degenfeld, G., Merchant, M. J., Blau, H. M., and Bogyo, M. (2007) Noninvasive optical imaging of cysteine protease activity using fluorescently quenched activity-based probes, *Nat Chem Biol 3*, 668-677.

10.   Lee, J., and Bogyo, M. (2010) Development of Near-Infrared Fluorophore (NIRF)-Labeled Activity-Based Probes for in Vivo Imaging of Legumain, *Acs Chem Biol 5*, 233-243.

11.   Hillaert, W., Verdoes, M., Florea, B. I., Saragliadis, N., Habets, K. L. L., Kuiper, J., Van Calenbergh, S., Ossendorp, F., van der Marel, G. A., Driessen, C., and Overkleeft, H. S. (2009) Receptor-Mediated Targeting of Cathepsins in Professional Antigen Presenting Cells, *Angew Chem Int Edit 48*, 1629-1632.

12.   Edgington, L. E., Berger, A. B., Blum, G., Albrow, V. E., Paulick, M. G., Lineberry, N., and Bogyo, M. (2009) Noninvasive optical imaging of apoptosis by caspase-targeted activity-based probes, *Nat Med 15*, 967-U177.

13.   Hu, M. Y., Li, L., Wu, H., Su, Y., Yang, P. Y., Uttamchandani, M., Xu, Q. H., and Yao, S. Q. (2011) Multicolor, One- and Two-Photon Imaging of Enzymatic Activities in Live Cells with Fluorescently Quenched Activity-Based Probes (qABPs), *J Am Chem Soc 133*, 12009-12020.

14.   Chang, J. T., Ciocca, M. L., Kinjyo, I., Palanivel, V. R., McClurkin, C. E., De Jong, C. S., Mooney, E. C., Kim, J. S., Steinel, N. C., Oliaro, J., Yin, C. C., Florea, B. I., Overkleeft, H. S., Berg, L. J., Russell, S. M., Koretzky, G. A., Jordan, M. S., and Reiner, S. L. (2011) Asymmetric Proteasome Segregation as a Mechanism for Unequal Partitioning of the Transcription Factor T-bet during T Lymphocyte Division, *Immunity 34*, 492-504.

15.   Yang, P. Y., Liu, K., Ngai, M. H., Lear, M. J., Wenk, M. R., and Yao, S. Q. (2010) Activity-Based Proteome Profiling of Potential Cellular Targets of Orlistat - An FDA-Approved Drug with Anti-Tumor Activities, *J Am Chem Soc 132*, 656-666.

16.   Bottcher, T., and Sieber, S. A. (2010) Showdomycin as a Versatile Chemical Tool for the Detection of Pathogenesis-Associated Enzymes in Bacteria, *J Am Chem Soc 132*, 6964-6972.

17.   Eirich, J., Orth, R., and Sieber, S. A. (2011) Unraveling the Protein Targets of Vancomycin in Living S. aureus and E. faecalis Cells, *J Am Chem Soc 133*, 12144-12153.

18.   Staub, I., and Sieber, S. A. (2009) beta-Lactam Probes As Selective Chemical-Proteomic Tools for the Identification and Functional Characterization of Resistance Associated Enzymes in MRSA, *J Am Chem Soc 131*, 6271-6276.

19.   Bachovchin, D. A., Brown, S. J., Rosen, H., and Cravatt, B. F. (2009) Identification of selective inhibitors of uncharacterized enzymes by high-throughput screening with fluorescent activity-based probes, *Nat Biotechnol 27*, 387-394.

20.   Patricelli, M. P., Nomanbhoy, T. K., Wu, J. Y., Brown, H., Zhou, D., Zhang, J. M., Jagannathan, S., Aban, A., Okerberg, E., Herring, C., Nordin, B., Weissig, H., Yang, Q. K., Lee, J. D., Gray, N. S., and Kozarich, J. W. (2011) In Situ Kinase Profiling Reveals Functionally Relevant Properties of Native Kinases, *Chem Biol 18*, 699-710.

21.   Patricelli, M. P., Szardenings, A. K., Liyanage, M., Nomanbhoy, T. K., Wu, M., Weissig, H., Aban, A., Chun, D., Tanner, S., and Kozarich, J. W. (2007) Functional interrogation of the kinome using nucleotide acyl phosphates, *Biochemistry-Us 46*, 350-358.

22. Kalesh, K. A., Sim, D. S. B., Wang, J. G., Liu, K., Lin, Q. S., and Yao, S. Q. (2010) Small molecule probes that target Abl kinase, *Chem Commun 46*, 1118-1120.

23. Nomura, D. K., Long, J. Z., Niessen, S., Hoover, H. S., Ng, S. W., and Cravatt, B. F. (2010) Monoacylglycerol Lipase Regulates a Fatty Acid Network that Promotes Cancer Pathogenesis, *Cell 140*, 49-61.

24. Ruckrich, T., Kraus, M., Gogel, J., Beck, A., Ovaa, H., Verdoes, M., Overkleeft, H. S., Kalbacher, H., and Driessen, C. (2009) Characterization of the ubiquitin-proteasome system in bortezomib-adapted cells, *Leukemia 23*, 1098-1105.

25. Arastu-Kapur, S., Ponder, E. L., Fonovic, U. P., Yeoh, S., Yuan, F., Fonovic, M., Grainger, M., Phillips, C. I., Powers, J. C., and Bogyo, M. (2008) Identification of proteases that regulate erythrocyte rupture by the malaria parasite Plasmodium falciparum, *Nat Chem Biol 4*, 203-213.

26. Hall, C. I., Reese, M. L., Weerapana, E., Child, M. A., Bowyer, P. W., Albrow, V. E., Haraldsen, J. D., Phillips, M. R., Sandoval, E. D., Ward, G. E., Cravatt, B. F., Boothroyd, J. C., and Bogyo, M. (2011) Chemical genetic screen identifies Toxoplasma DJ-1 as a regulator of parasite secretion, attachment, and invasion, *P Natl Acad Sci USA 108*, 10568-10573.

27. Harbut, M. B., Velmourougane, G., Dalal, S., Reiss, G., Whisstock, J. C., Onder, O., Brisson, D., McGowan, S., Klemba, M., and Greenbaum, D. C. (2011) Bestatin-based chemical biology strategy reveals distinct roles for malaria M1-and M17-family aminopeptidases, *P Natl Acad Sci USA 108*, E526-E534.

28. Horn, M., Nussbaumerova, M., Sanda, M., Kovarova, Z., Srba, J., Franta, Z., Sojka, D., Bogyo, M., Caffrey, C. R., Kopacek, P., and Mares, M. (2009) Hemoglobin Digestion in Blood-Feeding Ticks: Mapping a Multipeptidase Pathway by Functional Proteomics, *Chem Biol 16*, 1053-1063.

29. Blais, D. R., Lyn, R. K., Joyce, M. A., Rouleau, Y., Steenbergen, R., Barsby, N., Zhu, L. F., Pegoraro, A. F., Stolow, A., Tyrrell, D. L., and Pezacki, J. P. (2010) Activity-based Protein Profiling Identifies a Host Enzyme, Carboxylesterase 1, Which Is Differentially Active during Hepatitis C Virus Replication, *J Biol Chem 285*, 25602-25612.

30. Witte, M. D., Kallemeijn, W. W., Aten, J., Li, K. Y., Strijland, A., Donker-Koopman, W. E., van den Nieuwendijk, A. M. C. H., Bleijlevens, B., Kramer, G., Florea, B. I., Hooibrink, B., Hollak, C. E. M., Ottenhoff, R., Boot, R. G., van der Marel, G. A., Overkleeft, H. S., and Aerts, J. M. F. G. (2010) Ultrasensitive in situ visualization of active glucocerebrosidase molecules, *Nat Chem Biol 6*, 907-913.

31. Kaschani, F., Gu, C., Niessen, S., Hoover, H., Cravatt, B. F., and van der Hoorn, R. A. L. (2009) Diversity of Serine Hydrolase Activities of Unchallenged and Botrytis-infected Arabidopsis thaliana, *Mol Cell Proteomics 8*, 1082-1093.

32. Wang, Z., Gu, C., Colby, T., Shindo, T., Balamurugan, R., Waldmann, H., Kaiser, M., and van der Hoorn, R. A. L. (2008) beta-Lactone probes identify a papain-like peptide ligase in Arabidopsis thaliana, *Nat Chem Biol 4*, 557-563.