ICI institute for chemical immunology

Interview

THE UBIQUITIN FIELD HAS DIVERSIFIED AMAZINGLY

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When biochemist Benedikt Kessler became fascinated by the polypeptide ubiquitin in the late '90s, the field was quite a niche. Still, the biological functions of many ubiquitin processing enzymes, including half of the deubiquitylases (DUBs) that Kessler studies remain unknown. Today, however, ubiquitin DUBs are very *en vogue* as novel targets in human disease," says the Oxford University professor. "Not just in academia, but also in pharma."

You are the keynote speaker at the 2023 ICI Conference honouring Huib Ovaa. How did you two meet?

"Huib and I met at the laboratory of Hidde Ploegh at Harvard Medical School in Boston. That must have been around 2000. We were both working there as postdocs, studying the role of proteolysis in antigen processing and presentation. We've kept in contact ever since, cooperating in numerous studies and in European networks on deubiquitylating enzymes, a common topic. There still is a continuous exchange between people working here in Oxford and in Leiden.

How do you remember him?

"A most remarkable hallmark of Huib, I found his unique way of thinking. Your very first impression could be: what does this guy mean? What is he talking about? Because his ideas often came from a totally unexpected angle. Soon, however, you would realize that what he was proposing was actually an extremely clever idea. Our collaborations were often successful because of our different ways of thinking. He was ▶

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one of the most brilliant minds in chemistry and chemical biology that I've known."

When you started your studies into DUBs, it was quite unexplored territory. That has changed significantly, hasn't it?

"Yes and no. A lot of progress has been made, but even today we only know the biological function of a fraction of the approximately one hundred DUBs encoded in the human genome. A lot more research still needs to be done. Nevertheless, there is a considerable interest in DUBs, also from pharma. That's because DUBS are now recognised as viable drug targets. Also, there is interest in the DUBTAC approach [Deubiquitinase Targeting Chimera, ed.], a method that uses small molecules to recruit DUBs to deubiquitylate beneficial proteins such as tumour suppressors, in order to keep doing their good work. Molecular glues to degrade or stabilise therapeutically relevant proteins are very, very *en vogue* now. I'm currently on the board of a few companies and startups in the field."

"Chemical probes are essential tools in our lab"

Could this approach cause a revolution in immunology like the checkpoint inhibitors?

"It might. DUBs and the E3-ligases, the enzymes that add and remove ubiquitin, seem the new frontier in translational research in immunology, immune oncology and neuroinflammation. Both enzyme families are quite diverse which adds to their attractiveness. There are about seven hundred E3s encoded in the human genome and, as mentioned, about one hundred DUBs."

In which diseases are ubiquitin enzymes involved?

"In most types of human diseases. For instance, the ubiquitin E3-ligase Parkin and a DUB called USP30 have been linked to Parkinsons disease. They control mitophagy, the cell mechanism to remove defective mitochondria. Some small inhibitors of USP30 are already considered as potential clinical candidates for Parkinsons. Moreover, defective mitochondria are a major hallmark of other neurodegenerative diseases, but also of acute kidney disease and lung fibrosis."

Have you been surprised by this recent 'hype' about DUBs?

"Actually, yes. Today, the enzymes themselves attract considerable attention. When we started ubiquitin research was focused on degradation and antigen processing. Now, it's also about signalling and modification of function because ubiquitylation also targets proteins for differenT-cell locations. The ubiquitin system turned out to be far more complicated than we realized when we started experimenting in the lab of Hidde Ploegh. The field has diversified amazingly."

What's your research focus in the coming years?

"There are still more than seventy DUBs for which the biological role needs to be elucidated in more detail. It would be nice to do so, but we won't be able to do that alone. Lately, our focus has shifted towards the role of DUBs in intracellular signalling and also in innate immune signalling. We have, for example, a big project running about the role of DUBs in activating the inflammasome complex, a major form of innate immune signalling."

What role does chemistry have in your studies?

"We have a lot of cooperations with chemistry labs, but I also always have a chemist in the group. Chemical probes are essential tools in our lab, every master student learns how to synthesize at least one. We heavily use the HA ubiquitin propargyl probes invented in Huib's lab, for example. We call them HAUb, reminding us of Huib's substantial contribution. They are really remarkable, and *the* exception to the bio-orthogonal click chemistry developed by recent Nobel prize winners including Carolyn Bertozzi. When you attach them to the C-terminal of ubiquitin, they react beautifully with endogenous enzymes, with DUBs or E3 ligases."

What do you enjoy most in your work today?

"Looking at new research data with students and postdocs and interpret those. Making sense of the often very complex proteomic signatures. It's a big privilege to witness new scientific discoveries up close. It is actually a kind of an addiction; you are always looking forward to the next new result."

"It is a kind of addiction; you are always looking forward to the next new result"

Can you give a glimpse of what you will present at the coming ICI conference?

"My lecture will focus on an example of how ubiquitin intersects with immunity. It's about USP18. Although the name is short for ubiquitin-specific protease 18, it is actually *not* a DUB. USP18 does not recognize nor cleave ubiquitin, and believe me we have tried and tried. What it does cleave, we have realised, is a very close analogue of ubiquitin involved in the type I interferon response, which turns out to be a highly interesting therapeutic immune oncology target." ■

Commemorating to Huib Ovaa

It has been three years since a special man passed away. A great loss to science, especially to the chemical immunology community. A highly gifted, motivated, productive and above all a very creative scientist. He was able to look beyond the boundaries of his own field, chemistry, which allowed him to broaden his view. Add to this his fascination for biology and it is clear why he played such a prominent role in the development of a new and promising field Chemical Biology and in extension Chemical Immunology. This year's ICI conference is all about Huib and ICI Bulletin presents a special edition in his honor.

▲ ***From top:*** *Benedikt Kessler, Monique Mulder, Paul Geurink, Gerbrand van der Heden, Celia Berkers.*

Celebrating Huib's science

In the column Celia Berkers looks forward to celebrating Huib's science during the annual ICI conference. And to hear about DUBs and ubiquitin, about chemical toolboxes and probes, about green mice in Boston and pink hoods in Amsterdam and about all those scientific adventures that would not have been the same without Huib.

Benedikt Kessler

In the interview Kessler tells how he met Huib, how he had to learn to deal with him and how they then become best friends and colleagues with a common interest in deubiquitylating enzymes. "A most remarkable hallmark of Huib, I found his unique way of thinking."

Van der Heden & Mulder & Geurink labs

The former Ovaa lab is now under supervision of triumvirate: Gerbrand van der Heden van Noort, Monique Mulder and Paul Geurink. They continue Huib's work with their teams and build on the solid scientific foundation that he left behind. In the section Science three main papers are discussed that were published after Huib's death but in which his scientific impact is undeniable. The scientists involved miss him, not only because of his scientific insights, but also because of his humor. They use words like: "I think Huib would have had a good laugh about our approach" or "Huib was always triggered by unexpected" or "Huib would call this type of work 'rocket science'"

UbiQ

In the section Business Partner UbiQ speaks. The company was founded in 2010 as a spin-off of the Ovaa lab for establishing a broad toolbox of chemical reagents to study ubiquitin-proteasome system. It turned out to meet a need. "Business is running fine," tell cofounders Farid El Oualid and Alfred Nijkerk. But Huib is missed. "His passing has left a big mark on us and the research community."

Thermal peptide exchange technology

What started as an idea from the labs of Sjaak Neefjes and Huib Ovaa has now grown into a user-friendly method for screening immune cell responses to any antigen. In the section collaboration scientists involved explain how the planted seed grew into an elegant technology to study immune cells thanks to close collaboration between multiple research groups.

An unexpectedly successful route to ADPr modified ubiquitin

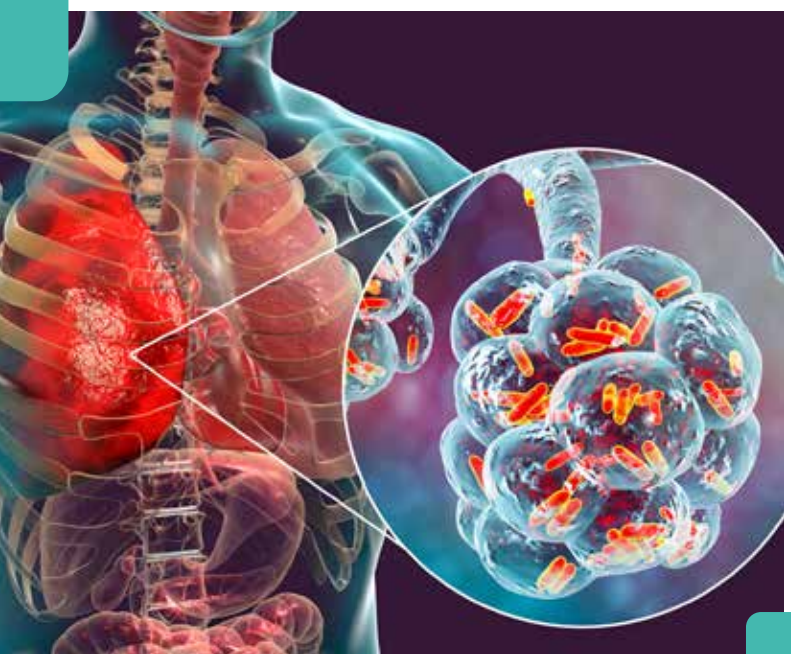
Through the synthesis of a series of modified ubiquitins, scientists at Leiden UMC could solve part of the puzzle of how *Legionella* hijacks its hosT-cells. "I think Huib would have had a good laugh about our approach."

To create an environment in which it can replicate most effectively, *Legionella* bacteria interfere with their hosT-cell's biochemistry. One pathway Legionella manipulates is the ubiquitin pathway, the system by which a cell marks proteins for destruction and starts up the immune system. The bacterium puts an adenosine-di-phosphate-ribosyl (ADPr) group on the hosT-cell's ubiquitin which leads to a cascade of follow-up reactions that allows the bacterium to dodge the immune system. Without this cunning trick *Legionella* replication is greatly reduced.
"It has always been assumed that Legionella attaches the ADPr-group specifically on Arg42 of ubiquitin, not on one of the other three arginines in the protein," says Gerbrand van der Heden van Noort, assistant professor at Leiden UMC. "However, it had never been proven."

Limits of current chemistry

Proof could come from synthesizing all four possible regioisomers of ADPr-ubiquitin (Ub^{ADPr}) and see which one(s) Legionella enzymes would accept as a substrate. However, there was a huge hurdle to overcome: synthesis of the regioisomers seemed impossible using 'classic' solid-phase peptide synthesis. PhD-student Max Kloet: "Synthesizing ubiquitin using solid-phase chemistry is no problem, however, introducing a modified arginine on which we could couple the ADPr-group pushes the limits of current chemistry." The even bigger problem comes in the final step: release of the product from the resin. That is usually done by adding a strong acid, but the glycosidic bonds in the ADPr-group, and also the pyrophosphate group are known to be hydrolysed at low pH.

▼ *Legionella bacteria can cause a serious type of pneumonia (lung infection) called Legionnaires' disease. The Van der Heden team unravelled cellular pathways of how the bacterium hijacks its hosT-cell. They found that the bacterium puts an adenosine-di-phosphate-ribosyl (ADPr) group on the hosT-cell's ubiquitin which leads to a cascade of follow-up reactions that allows the bacterium to dodge the immune system.*



> ***"The fastest way to know what a compound can withstand, is to just destroy it"***

Kloet: "Most likely, if we could synthesize the regioisomers on the resin, we would never be able to cleave them of intact."
Van der Heden van Noort: "It was Huib Ovaa who suggested to just try it anyway, which is typically Huib." And that is what the scientists did. Fortunately, Ub^{ADPr} turned out to be far more robust than one would expect. The very first time Kloet released a newly synthesized Ub^{ADPr} regioisomer from the resin, it came off quite undamaged. Kloet: "Often the fastest way to know what a compound can withstand, is to just destroy it."
After optimizing the decoupling conditions, all four different regioisomers could be synthesized. The rather surprising stability of Ub^{ADPr} in strong acid may also open up a new synthetic route towards other glycosylated proteins. Van der Heden van Noort: "I think Huib would have had a good laugh about it, if he could have seen the results."

Collaboration pays off

Of the four regioisomers indeed only the one with ADPr at Arg42 was accepted by Legionella enzymes as a substrate. The enzymes for this study were provided by the group of Professor Ivan Đikíc at Goethe University Frankfurt which specialises in molecular signalling and was amongst the first to identify Arg42 ADPribosylation of ubiquitin by Legionella.
Van der Heden van Noort: "It was Huib who initiated the first contacts between our research groups. He strongly believed that cooperations can lift your work to a higher level."
Synthetic Ub^{ADP} can also be used as a tool to discover if other pathogens use the 'Legionella-trick' to influence their hosT-cell, emphasizes Kloet. It is now used as a probe to 'catch' such bacterial enzymes. "Perhaps also human cells use this pathway under certain conditions," adds Van der Heden van Noort. "With this new tool we will certainly study that, too. In fact, we have started already." ■

Reference

Jim Voorneveld, Max Kloet, *et al.* **Arginine ADP-Ribosylation: chemical synthesis of post-translationally modified ubiquitin proteins'** JACS 2022, 144, 20582 doi.org/10.1021/jacs.2c06249

Seeing is believing: a super assembly transfers ubiquitin

Cryo-electron microscopy proved that many proteins get marked by a ubiquitin group by multiprotein super assemblies. Chemical probes made in Huib Ovaa's group in Leiden played an important role.

"For more than fifteen years, Huib and I discussed how to reveal the structure of ubiquitinating super assemblies," says Brenda Schulman, professor of Molecular Machines and Signalling at the Max Planck Institute of Biochemistry, Germany. "Actually, seeing such an assembly using cryo-electron microscopy was truly amazing, a wild dream come true. The structure is very beautiful, and once you've figured it out, very logical too. It's so sad that Huib was too sick to witness that moment."

A mystery

Adding a ubiquitin peptide marks proteins for destruction and regulates virtually all eukaryotic cellular pathways, such as cell division, transcription and signalling. Ten to twenty percent of all ubiquitination is thought to be performed by complex multiprotein assemblies. Despite their biological importance, the structure of these super assemblies remained a mystery for a long time.

The protein ARIH1 plays a central role in one type of assembly, named SCF-RBR E3-E3. ARIH1, a E3 ligase, transfers ubiquitin to one of approximately a thousand possible protein substrates of another E3, hence the term E3-E3. ARIH1 is essential for human cell survival, but completely inactive on its own. Its active site is blocked by another part of the protein, making it almost impossible to study.

Using various chemical probes and numerous biochemical techniques, Schulman and co-workers studied ARHI1 in partial and total super assemblies. In a paper in Cell in 2016, the team already proposed a 'draft' of the total structure. Schulman: "That paper, however, met considerable skepticism."

Because seeing is believing, the scientists turned to cryo-electron microscopy to test their hypothesis and succeeded in making snapshots of the assembly at various stages of the ubiquitination using both chemical and semi-synthetic peptide probes. The structures were published in a 2021 Nature paper.

In the super assembly, ARIH1 partners up with at least nine proteins, and is 'pushed' into a highly different conformation opening up the catalytic site that transfers ubiquitin to a selected protein substrate bound to the SCF E3 ligase. Each super assembly consists of two E3 ligases and the substrate is delivered by one of seventy possible F-box proteins that all bind a range of proteins. This mix-and-match system makes the super assembly a highly versatile ubiquitination 'machine'.

No certainty in biology

Monique Mulder, associate professor LUMC, contributed to the project by designing and synthesizing synthetic probes: "We finished our probes in 2017. They were an important, but only a first step in this huge, enjoyable project. Brenda's enthusiasm is a real driving force, and I'm very proud that our labs still have a very active collaboration. Huib would call this type of work 'rocket science' and I am sure this Nature paper would have put his signature big smile on his face."

Huib inspired this research and provided excellent research tools, emphasizes Schulman. "We lost an excellent scientist, but I also lost a very dear friend. Being a blunt Dutchman, he was one I could trust to tell me when I was on the wrong path, too."

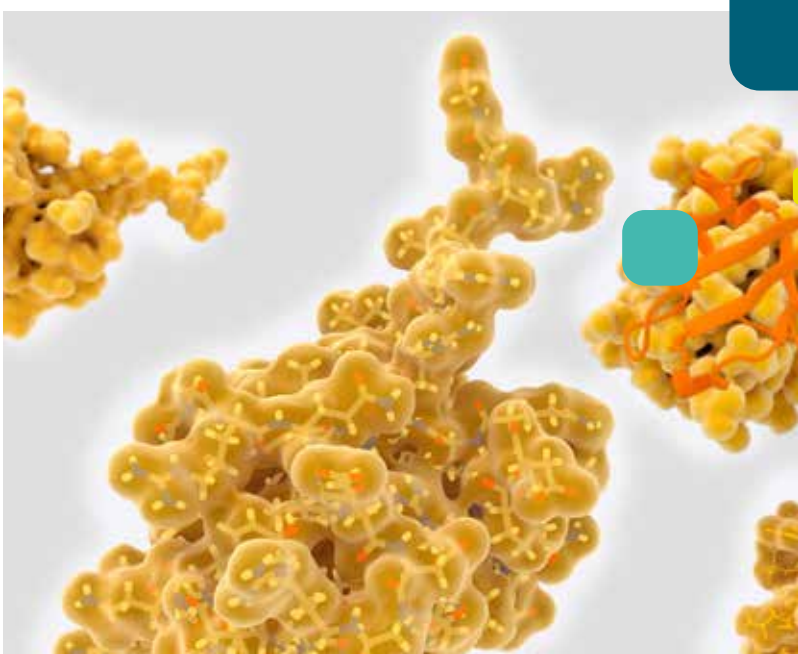
> ***"Huib would call this type of work: rocket science"***

How sure is Schulman that the presented super assembly indeed captures the true biological structure of this huge complexes? "There is no certainty in biology. We tested our hypothesis and thus far it stands. Being a good scientist means that we continuously keep looking for contradictory signals, also because the unexpected is always the most exciting!" ■

References

Daniel Horn-Ghetko *et al.* **Ubiquitin ligation to F-box protein targets by SCF-RBR E3-E3 super-assembly**, Nature 2021, 590, 671, doi.org/10.1038/s41586-021-03197-9

▼ *Ubiquitin (different views of its structure are shown) is a small peptide that can be conjugated to a protein. Ubiquinated proteins play a main role in regulating of cellular pathways such as division, transciption and signalling. Leiden scientists have been challenged for many years to reveal the structure of ubiquitinating super-assemblies. Ultimately, cryo-electron microscopy offered the solution. The Mulder team designed and synthesized needed probes contributing to the first steps in this huge process.*



A surprise hit results in probe for PARK7-research

Studying deubiquitinating enzymes, scientists at Leiden UMC stumbled upon an interesting off-target protein: PARK7, involved in Parkinsons disease, but also in cancer. It resulted in a toolbox for studying its activities.

Checking the specificity of a new probe for the deubiquitinating enzyme UCHL1, assistant professor Paul Geurink and senior scientist Aysegul Sapmaz of the Leiden Department of Cell and Chemical Biology found a surprising off-target protein. Their probe also reacted with PARK7, human Parkinson disease protein 7.
UCHL1 and PARK7 have totally different biological functions. UCHL1 cleaves of ubiquitins, primary involved in protein degradation. It is a member of the DUBs-family, a central topic in the research group. PARK7 is implicated in Parkinsons disease. Mutations in its gene are a major risk factor for this infamous neurodegenerative disease.

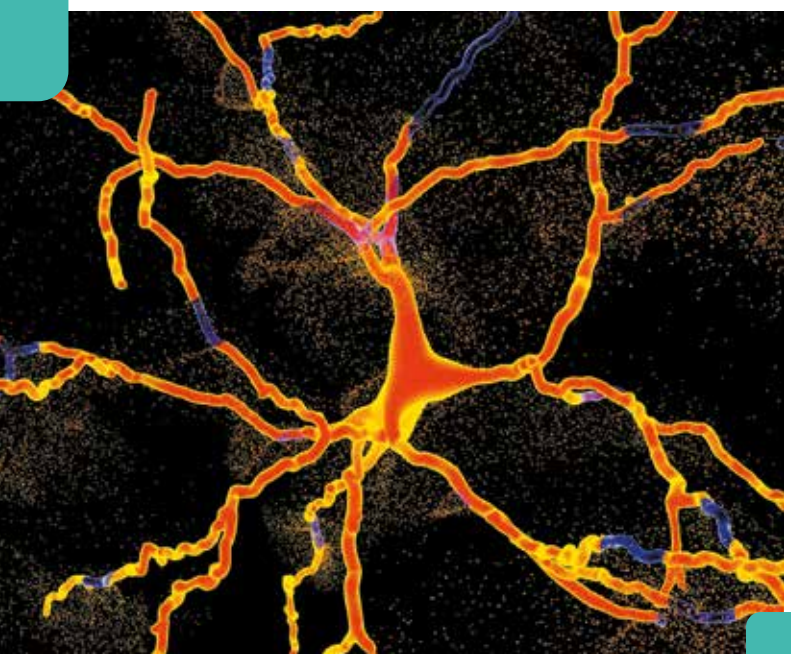
Pioneering work

Geurink: "That PARK7 popped up in our assays was quite a surprise, but both proteins have a central cysteine in their catalytic pocket that reacts with the probe." Huib Ovaa, group leader at the time, was immediately intrigued by the finding, and because a probe for measuring PARK7 activity was not available yet, the scientists proposed to develop one, starting from the UCHL1-probe. A good idea, agreed Ovaa. Geurink: "Huib was always triggered by the unexpected."
PhD-student Yuqing Jia (now a postdoc at ETH, Zurich) took up the challenge to develop a cell-permeable, fluorescent probe to monitor PARK7 activity. Truly pioneering work as no useful assay for the protein was known. Jia discovered that the enantiomer of the original UCH1-probe, was a highly selective PARK7 inhibitor, and synthesized two probes with different fluorescent labels. One (Rhodamine-labelled) is suited for high-throughput screening, the other (SulfoCy5-labelled) is useful for detecting active PARK7 in cell lysates.

> *"PARK7 is a potential biomarker to detect cancer in urine or blood"*

To showcase the toolbox, the scientists screened a library containing nearly eight thousand small molecules for PARK7 inhibitors using the Rhodamine probe, cherry-picked three potent hits and validated them using the SulfoCy5-probe in a cell lysate assay.
Geurink: "The paper shows that we've developed the tools to determine PARK7-activity and find inhibitors." Inhibitors of PARK7 may be of interest in cancer diagnostics, because, more recently, it has become evident that upregulation of the protein is associated with various types of cancer and with chemoresistance in cancer therapy.

▼ *Decline of dopaminergic neuron is an important stage in the development of Parkinsons disease. PARK7 plays a key role in this disease. The Geurink team developed a new probe for the deubiquitinating enzyme UCHL1 and they accidentally discovered that their chemical tool also reacts to PARK 7. This discovery may lead to a promising toolbox for early diagnosis and therapy monitoring.*



Promising chemical tools

Sapmaz: "Tools based on PARK& inhibitors could for example be useful in the early diagnosis of cancer or for monitoring cancer therapy. PARK7 is excreted by certain cancer cells and thus a potential biomarker in urine or blood." "Actually," Sapmaz continues, "we've already discovered some potent PARK7-inhibitors in follow-up experiments using our toolbox. Some may be used to create proteolysis-targeting chimeras, PROTACs, a hot topic in pharma today. We are writing a follow-up paper."
When the toolbox indeed leads to new cancer diagnostics, Huib Ovaa will have contributed to the fight against the disease that caused his death. Geurink: "It's perhaps a bit of a weird link, but precisely in line with Huib's mission: making chemical tools to help solve biological questions." ■

Reference

Yuqing Jia *et al.* **Chemical Toolkit for PARK7: Potent, Selective, and High-Throughput**, J. Med. Chem. 2022, 65, 13288, doi. org/101021/acs.j.medchem.2c01113

A CHEMICAL APPROACH TO SOLVING BIOLOGICAL QUESTIONS

UbiQ was founded in 2010 as a spin-off of the Ovaa lab at the Netherlands Cancer Institute. The small company develops reagents for ubiquitin research and is involved occasionally in drug discovery projects, says co-founder Farid El Oualid. "Our mission is ultimately to help patients. With UbiQ, we aim to explore all possible drug targets using our technology, and to facilitate others to do their research by providing reagents."

A pile of FedEx envelopes is stacked on a desk in the small UbiQ office. The company has just moved to the shiny new, fully sustainable Matrix One building at the Amsterdam Science Park. One envelope is filled with a sample and ready to be sent. "Yes, we do this ourselves," smiles Farid El Oualid, co-founder and CSO of UbiQ.
El Oualid runs the company, co-founded by Huib Ovaa, together with Alfred Nijkerk, who is responsible for the business and financial part of UbiQ. He has a solid background in pharmaceutical industry and as an entrepreneur in life sciences. El Oualid carries out the lab work including proof-of-concept experiments and synthesising reagents. There is no other permanent staff on the team. Another smile: "Yes, I answer the phone for UbiQ as well. I found that our customers appreciate it to get a quick solution to their questions." On top of this El Oualid is a senior scientist at the Netherlands Cancer Institute (NKI).

An omnipresent protein

Ubiquitin is a small signalling protein found in most tissues of eukaryotic organisms. Binding of ubiquitin to another protein can have many effects varying from changing its activity or interactions to marking it for degradation.
To investigate the function of ubiquitin, reagents are needed. This can be anything from a fluorescent probe to inhibitors of ubiquitin activating enzymes and also ubiquitin-like proteins, chains or ubiquitinated peptides. Using technologies developed by Huib Ovaa and Farid El Oualid, UbiQ has taken a chemical approach in developing these reagents to solve biological questions.

Originally, all work was done at the Ovaa-lab which was part of the NKI in Amsterdam at the time. In 2016, the Ovaa-lab moved to the LUMC in Leiden, which led to a natural way for Huib Ovaa to concentrate more on his research and less on the company. "Huib was an academic scholar at heart," El Oualid says. "We also felt it was sensible to separate commercial and academic activities to prevent any conflict of interest." El Oualid and Nijkerk continued to run the company on a daily basis with regular work discussions with the Ovaa-group, NKI and many others. "Huib's passing has left a big mark on us and the research community as well, but fortunately UbiQ was able to keep running as before," says El Oualid.

Mission and vision

The mission and vision of UbiQ hasn't changed much over time, says El Oualid. The core of the work is designing and synthesizing reagents for others to use. "Our strength lies in these basics and in the fact that we can provide solid, freeze-dried samples of our proteins, so our customers don't have to worry about storage and stability."
The second part of the company strategy is drug discovery, from concept or idea to designing reagents critical for the developing process. "Drug discovery projects are not always successful, but we learn a lot about the process." In the near future, UbiQ wants to continue to work on reagents that are more elusive and have not been developed yet, says El Oualid. "A great challenge, but each step forward will be significant for the drug discovery community worldwide." ■

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An elegant technology to study immune cells

What started as an idea from the labs of Sjaak Neefjes and Huib Ovaa has now grown into a user-friendly method for screening immune cell responses to any antigen. The success is based on collaboration between multiple research groups.

Thermal peptide exchange technology. The term sounds like a simple lab protocol. But at a closer look it's a perfect example of multidisciplinary, multi-group research where a fundamental background, a good idea and an interest in technology lead to new insights and relevant medical applications.

The field of major histocompatibility complexes has long been a bit of a neglected child. Sjaak Neefjes and Huib Ovaa had an interest in the field though, both working at the NKI in Amsterdam about a decade ago. With several collaborations between the groups already ongoing, Ferenc Scheeren, group leader at the department of Dermatology at LUMC, also became involved in the work on MHC-I. He says: "I had an interest in technology, just like Huib. We had a good relationship at work and in private and we received a large grant together. The work on MHC was a small part of Huib's research, but it got a lot of love from him."

A conserved target

As part of this interest in MHCs, Scheeren and Ovaa started a collaboration with Paula Ruibal and Simone Joosten at the department of Infectious diseases at LUMC, aimed at vaccine development against infection with Mycobacterium tuberculosis (Mtb), including technology development such as thermal peptide exchange. After a challenging few year, in which the labs were not functioning as normal because of the COVID-19 pandemic and on top of that the passing of Huib, Neefjes asked Scheeren to take over the MHC research from the Ovaa-lab, including the project on thermal peptide exchange. Added to the group was Ian Derksen, who had been developing the method in the Ovaa-lab under Jolien Luimstra's supervision. As an expert in the technique, he continued his work under supervision of Scheeren and Ruibal.

Instead of the most studied classical MHC-I allele HLA-A*02:01, Ruibal's group studied peptides that bind to the non-classical HLA-E to investigate immune responses against an Mtb infection. Ruibal explains: "While HLA-A*02:01 is most abundant in the Caucasian population, HLA-A molecules are genetically highly diverse. HLA-E is much more conserved between people. Most people express the same alleles; we know two alleles which differ by only one mutation. That means if you find an antigen or peptide that you, for instance, want to base a vaccine on, we expect that most of the population has the same response and thus that the vaccine will be effective for most people."

Studying a different MHC-I allele meant that Derksen had to re-design his peptide exchange method to work for HLA-E. He

"The discovery of a new peptide template was real serendipity"

explains: "First, we needed to find a new template peptide. Finding it was real serendipity. I received a peptide which had a synthetic error in it: one of the amino acids was accidentally added to the original peptide. But this one turned out to be the best." Then he had to adapt the method for thermal

exchange. “This was difficult. Everything with HLA-E is more difficult. We managed to exchange 80% of the peptides in the best case, whereas for HLA-A*02:01 you can exchange more than 95%.” Derksen found a smart workaround for this: instead of first making HLA-E tetramers and then performing the thermal peptide exchange, he performed the thermal exchange using HLA-E monomers and then made the tetramers. In this way the tetramers, which are used for the immunoassays, are loaded with the peptide of interest at similar rates to those of HLA-A*02:01.

Easy-to-use platform

The efforts have led to a greater understanding about producing MHC-I-peptide complexes, which the researchers want to share with the world. Sjaak Neefjes, Huib Ovaa together with Malgorzata Garstka and Jolien Luimstra have filed a patent for the technology. Importantly, using this technology, MHC-I-tetramers with a given peptide can very easily be produced. Clinical researchers with no specific expertise can then use these in immunoassays, such as Ruibal is doing. “It is suitable as a high-throughput technology, which we can use to screen many peptides and select the best to further investigate immune responses that could be involved in a potential vaccine.”

Scheeren believes the technology can help to design better vaccines. “To fully engineer a vaccine, you need to know the dominant T-cell epitopes; the peptides that are presented by the MHC and recognised by T-cells. This exchange technology makes it possible to go there. That is why we developed the platform.”

> *“The technology can help to design better vaccines”*

Scheeren meanwhile is looking ahead to expand the technology. “I look forward to completing the projects Huib started and then to stand on his shoulders and move on. One thing we are working on is the development of a quality control tool for thermal peptide exchange. Additionally, I think it is important to expand the technology to include more diversity. HLA-A*02:01 is a predominantly Caucasian phenotype. I want to include a wide range of alleles within the MHC field to identify peptide antigens in people with different geographic backgrounds.” ■

Thermal peptide exchange technology

Major histocompatibility complexes, or MHCs, mediate an ingenious part of the adaptive immune system. Class I MHCs, expressed on the surface of nucleated cells, have one purpose: to present fragments (peptides) from proteins synthesised within the cell to cytotoxic (killer) T-cells on the cell surface. There, killer T-cells scan these peptides by binding to the peptide-MHC-complex. If the peptide antigens are foreign, for example originating from a virus or tumour, the infected or mutated cell is destroyed and the antigen-specific T-cells proliferate in search of other affected cells. The ability of adaptive immune cells to distinguish between self and foreign proteins is essential. If the process fails, this can lead to autoimmunity.

Monitoring the presence of antigen-specific T-cells can provide information about, for example, a patient’s immune system and former infections. T-cell specificities can be investigated using MHC-I tetramers loaded with a specific antigenic peptide, for example originating from a melanoma cell from a patient. Therapies can then include isolation and expansion of these T-cells and re-introducing them to boost the immune reaction to affected cells.

But producing and purifying MHC-I-peptide complexes is a tedious process that takes days per peptide of interest. An easier way is to produce one batch of tetramers loaded with a so-called template peptide, which can be exchanged for a peptide of choice. An example of an established method employs a photocleavable peptide that can be exchanged by exposure to UV. This technology has some drawbacks, however, including the sensitivity of biomolecules to UV radiation. In the lab of Sjaak Neefjes, the idea of using differences in thermal stability to facilitate exchange was born.

For this thermal peptide exchange, MHC-I tetramers are complexed with a template peptide, selected for its ability to form a complex that is stable at low temperatures (preferably below 10°C), but dissociates at higher temperatures (ideally room temperature). The complexes of conditional MHC-I tetramers can be exchanged for any peptide of interest that binds more stably at higher temperatures than the template peptide. In short, template-MHC-I-complexes are mixed with a solution containing a new peptide of interest and heated to room temperature, thus exchanging the peptides and forming new complexes in a quite simple, time-effective manner. This facilitates the use of high-throughput methods for example to quickly scan many MHC-I-peptide complexes for T-cell recognition.

In a close collaboration, former PhD student Jolien Luimstra and student Ian Derksen, both working in the Ovaa-lab at that moment, carried out the research. NKI and LUMC have filed a patent application for the temperature-mediated peptide exchange technology.

Finding the Huib32 inhibitor

It's an unconventional ICI-project in many ways. LUMC researcher Aysegul Sapmaz wrote the proposal to study ubiquitylation of Rab-proteins back in 2019 together with Huib Ovaa and Sjaak Neefjes. She then moved on to do the research herself in close collaboration with PhD student, Esther ter Linden from the Neefjes group.

Aysegul Sapmaz moved to Ovaa's group at the NKI after finishing her PhD as a molecular biologist at the Middle East Technical University in Ankara, Turkey. The move to NKI was no coincidence, Sapmaz tells, as she already joined the Ovaa group as a visiting PhD student at the latest stage of her PhD research. "As a biologist, I find it magical to combine chemistry and biology here. We bring out so many cool ideas together." One of these ideas was to study the reversible ubiquitylation of Rab-proteins in the context of endosomal trafficking and immunity. Based on published data from the collaboration of the Ovaa and Neefjes groups and some preliminary chemical experiments, Sapmaz wrote a proposal for this ICI project with Huib Ovaa and Sjaak Neefjes, which was granted in 2019.

Searching for the missing link

Rab GTPases are key regulators of various steps in the endo-lysosomal system. They are critical in a number of stages of antigen presentation. Rab7 is such a GTPase. The Ovaa and Neefjes labs have recently described how reversible ubiquitylation of Rab7 controls the endo-lysosomal system. Although they found that reversible ubiquitylation of Rab7 is controlled by a deubiquitinase called USP32, so far, it had remained unclear what the specific ligase is. Therefore, the project aimed to identify the ligase responsible for Rab GTPase ubiquitylation. "We think that this E3 ligase will provide targets for small molecule-mediated modulation of the immune response," explains Sapmaz.

Aysegul Sapmaz (senior scientist)
Cell and Chemical Biology, LUMC

Another aim was to find small molecules that can target the enzymes that are modulating the reversible ubiquitylation of Rab GTPases and can inhibit this process. Finding such an inhibitor would allow researchers to investigate the mechanism of the process and identify targets for immunotherapy.

Bridge between chemistry and biology

Initially, Sapmaz and PhD student Esther ter Linden in the Sjaak Neefjes group started on the project. "After Huib's passing, I moved on to mentoring more PhD students, and most lab work was done by one of my PhD students," she recalls.

With the project in its final stages this summer, most targets have been met. "We have identified a potential E3 ligase," says Sapmaz. "It is a good candidate for a drug target, but we are still validating it. After validating and characterizing our small molecule compound targeting USP32 in a simple system, we are also studying the turnover of the T-cell receptors and the release of cytolytic granules by activated cytotoxic T-cells, and we hope to see the effect of our compound."

"How chemistry can help understand biology and vice versa"

But the best result was finding a small molecule that inhibits USP32, says Sapmaz. "We have really impressive data from proteomics, cell-based experiments, and biochemistry. We have been able to combine our expertise in chemistry and biology to find this really potent inhibitor." The researchers have named the inhibitor Huib32 (Human deUbiquitinase Inhibitor 32).

Sapmaz stresses the collaboration between the former Ovaa lab and the Neefjes lab. "I feel like a bridge between chemistry and biology, as I work in both labs. The project shows how chemistry can help understand biology and vice versa." She has just received a grant from NWO to follow up this research, studying the role of de-ubiquitinating enzymes in lysosomal dysfunction in neurodegeneration illnesses such as dementia. ■

Project: Finding and manipulating ubiquitylation of Rab GTPases in endosomal traffic and immunity.

Antigen-specific B-cell targeting in rheumatoid arthritis

A major challenge for treatment of rheumatoid arthritis is the selective elimination of pathogenic autoreactive B-cells while retaining protective B-cells. PhD student Kevin Venrooij focuses on the use of synthetic autoantigens to tackle this problem.

Rheumatoid arthritis (RA), one of most prevalent autoimmune diseases, cannot be cured but is remarkably responsive to therapeutic depletion of B-cells. Unfortunately, current B-cell targeted strategies kill both protective and pathogenic B-cells, leading to immune deficiency. Depletion of only pathogenic, i.e., auto-reactive, B-cells would provide a solution. However, that is not an easy task, knows Kevin Venrooij, who dedicates his PhD research to selective B-cell targeted treatment of RA.
He is a PhD student at the synthetic organic chemistry group of the Radboud University Nijmegen, where he also performed his master's internship. "At the time I participated in developing chemical tools for improving RA therapy strategies," Venrooij explains. "The research really appealed to me, and I wanted to continue working in this field after completing my master's degree *Molecular Life Sciences*." With the start of his PhD studies in 2019, his wish came true. In a sense, he is an outsider in the ICI PhD student group, since he is working 'solo' without a duo PhD partner. "But that works fine," he says. "As a molecular life scientist, I am familiar with chemistry and biology, so I understand both worlds."

"We use click chemistry to improve the CAAR T-cells even further"

Trojan horses

Bruton's tyrosine kinase (BTK) inhibitors disrupt crucial intracellular signaling in B-cells, which leads to breakdown of the cell. Therefore, BTK inhibitors are used to treat RA. "But you don't want to destroy all B-cells, just the pathogenic ones," Venrooij explains. One way to achieve selectivity is to develop Antigen Drug Conjugates. "We use chemical tool to develop such conjugates, which consist of a cleavable linker to which a synthetic autoantigen is linked on one side and a BTK inhibitor on the other. The coupled autoantigen leads to the right B-cell and after interaction with the B-cell receptor the conjugate internalizes. Subsequently, the payload drug is released in a Trojan horse like strategy."
Another way to achieve B-cell selectivity is using living drugs like chimeric autoantibody receptor (CAAR) T-cells. This approach is based on CAR T-cells, which are already being successfully applied in cancer immunotherapy. "Instead of antigen receptors, as in CARs, T-cells are genetically engineered to produce autoantibody receptors. Such CAARs specifically recognize B-cells secreting pathogenic autoantibodies after which these autoreactive B-cells are killed selectively by the T-cells," Venrooij clarifies. He focusses on click chemistry approaches to improve the CAAR T-cells even further. "We succeeded in designing a CAAR T platform where the engineered receptor can be labelled to contain any non-natural antigen. The platform is based on a self-labeling enzyme, which we functionalized with a citrulline-containing autoantigen." Venrooij works together with specialists in engineered CAAR T-cells at the University of Pennsylvania.

Own path

Finally, Venrooij talks about a third approach, so called photodynamic therapy (PDT), to achieve selective RA treatment. In this field he collaborates with the experimental rheumatology department of Radboudumc. "We have designed a small synthetic autoantigen construct equipped with a photosensitizer. The autoreactive B-cells are primed for destruction with 690 nm light within a minute of adding the construct. Thereafter, locally produced reactive oxygen species will kill the targeted cells," he tells.
Overall, Venrooij follows his own PhD path. He builds on previous ICI PhD projects in the field of B-cell targeting which he expands with new perspectives. Nevertheless, he is incredibly happy with the ICI PhD-network. "It is really fun and instructive to spar with each other about our research and to develop innovative ideas together. I also would not like to miss the PhD training program. For example, I learned how to make a good poster." And with success, last year he became ICI poster winner. ■

Kevin Venrooij
Radboud University
Chemical Immunology and Targeted Drug Delivery
Kimberly Bonger group

Column

ROCKET SCIENCE

CELIA BERKERS
ICI EXECUTIVE ADVISORY BOARD

Celia Berkers is professor of metabolomics at Utrecht University

Some people truly change our way of thinking. And often, you only really realize this afterwards. For me, Huib was such a person. And I imagine I am not the only one. Now, almost three years after his passing, he still sometimes pops up in my thoughts, or appears in a crazy dream. Because Huib was one of a kind. His Science was the one of 'whatever works', unconventional and, above all, fun!

Fun and amazing and ground-breaking Science, which Huib used to call 'Rocket Science', effortlessly went hand in hand in his lab. Huib inspired and ignited creativity in the people who worked for him. No idea was ever too crazy, no goal was ever out of reach. As a PhD student, there seemed to be endless freedom to just pursue my own ideas. If I would have to name one thing that still inspires me, it is this atmosphere. It also is the one lesson I try to convey to my own PhD students: that Science is about fun and freedom - a PhD is their time to shine.

Huib was wary of conventions and, above all, fearless. Why use an acoustic dispenser only for pipetting if you can also do combinatorial synthesis with it? Why going through all the trouble of expressing ubiquitin if you can also just synthesise it using a peptide synthesizer? When Huib was enthusiastic about something, he would find a way to make it happen, and often it did happen. It is this spirit that is still very much alive in the Ovaa lab today - now the Van der Heden & Mulder & Geurink labs. And I truly believe he would have loved to see his legacy alive and kicking!

I always look forward to our annual ICI conference, but as this edition is in light of commemorating Huib, it is extra special to me. So, I look forward to celebrating Huib's Science, to hearing about DUBs and ubiquitin, about chemical toolboxes and probes, about green mice in Boston and pink hoods in Amsterdam, and about all those scientific adventures that would not have been the same without Huib. Because some people truly change our way of thinking. See you all at the conference! ■

About ICI

The Institute for Chemical Immunology (ICI) is a Gravitation project, made possible by the Ministry of Education, Culture and Science, in collaboration with the Netherlands Organization for Scientific Research (NWO). ICI will define and exploit a new field termed chemical immunology and train a novel generation of interdisciplinary scientists. It aims to promote academic excellence. The ICI publishes twice-yearly the 'ICI Bulletin' featuring ICI research, education and other relevant developments. To (un)subscribe please send an email to info@chemicalimmunology.nl.

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