P53 CHAPERONING BY HSP90, HSP70, AND CO-CHAPERONES

This research is predominantly focused on the role of molecular chaperones in mammalian cells, including cell transformation. Previously, using highly purified recombinant human proteins, we have identified intermediate reactions that lead to the assembly of molecular chaperone complexes with wild type or mutant p53 tumor suppressor protein. We have discovered that Hsp90 exhibits higher affinity towards wild type p53 than to the conformational mutant p53. Lately we have demonstrated that Hsp90 molecular chaperone is required for binding wt p53 to the promoter sequences under physiological temperature of 37°C in an ATP-dependent reaction. These results obtained *in vitro* were supported by the observation that the treatment of human fibroblasts with geldanamycin or radicicol (Hsp90 specific inhibitors) resulted in a dramatic decrease of the p21 mRNA and, consequently, the p21 protein level, while the p53 mRNA and Ser-15P-p53 protein levels were mostly unaffected. Additionally, using Chromatin immunoprecipitation ChIP technology and realtime PCR, we showed that Hsp90 inhibitors decreased the amount of chromatin-bound p53 located near the p21/waf1 promoter sequence. Moreover, using *in vivo* FRET analysis, we showed that p53 forms a transient complex with Hsp90 and using DNA chip technology, and we likewise showed that transcription from other p53-dependent promoters is also affected by Hsp90 inhibitors.

In subsequent studies we have shown that at the physiological temperature of 37°C, Hsp90 molecular chaperone alone efficiently rescues the wild-type p53 promoter-DNA binding activity from thermal inactivation. At heat shock conditions of 40°C, the Hsp70 and Hsp40 chaperone machine is required to rescue the wild-type p53. At this temperature, Hsp90 stimulates the reaction by a limited direct action on wildtype p53, and by an indirect chaperoning which requires the presence of Hsp70, Hsp40, and Hop. Thus, for the first time we were able to distinguish two different modes of wt Hsp90 activity on a single substrate. Moreover, the Hsp90β E42A variant, despite its inability to hydrolyze ATP, is more efficient in the direct p53 rescue.

Using optimal molecular chaperone variants and reaction conditions (wt or E42A Hsp90β, Hsc70, Hdj1 or Hdj2 and Hop) we tested the ability of molecular chaperones to restore the promoter DNA binding of mutant p53 variants (R175H, R248Q, R249S, and R273H). Limited, but detectable recovery was observed mainly in the case of R249S p53. The analogies between wt and mutant rescue reaction requirements suggest that molecular chaperones stably associate with mutant p53 variants as a consequence of an attempt to refold these proteins. We have proven that MDM2 E3 ligase, in the absence of the E2 and E1 ubiquitylation system, can substitute for the Hsp90 molecular chaperone in promoting ATP-dependent binding of p53 to the p21/waf1 promoter-derived sequence. We have shown that the ATP-binding mutant MDM2 protein (K454A)

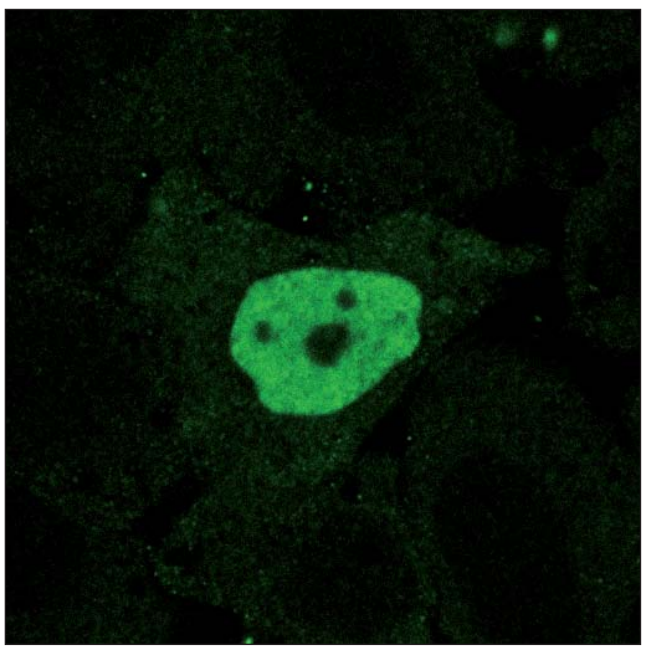

Fig. 1. HeLa cells were transfected with p53 and subjected to 42°C heatshock, fixed, and stained for p53. The depicted cell exhibits nuclear localization of p53, indicating that transient heatshock is not a factor efficiently changing intracellular transport of wild-type p53. (M. Olszewski)

lacks the chaperone activity both *in vivo* and *in vitro*. MDM2 co-transfected with wild-type p53 stimulates efficient p53 protein folding *in vivo* and this effect is abrogated when the ATP-binding defective form of MDM2 is used. This is the first demonstration that MDM2, in which overexpression is a new independent factor of adverse prognosis in non-small cell lung cancer, possesses an intrinsic molecular chaperone activity and indicates that the ATP-binding function of MDM2 can mediate its chaperone function towards p53 tumor suppressor. It was reported previously that MDM2 interacts with, but does not ubiquitylate, several transcription factors, which could affect cell transformation. Our findings that MDM2 is a novel molecular chaperone could help to explain the p53-independent oncogenic activity of MDM2.

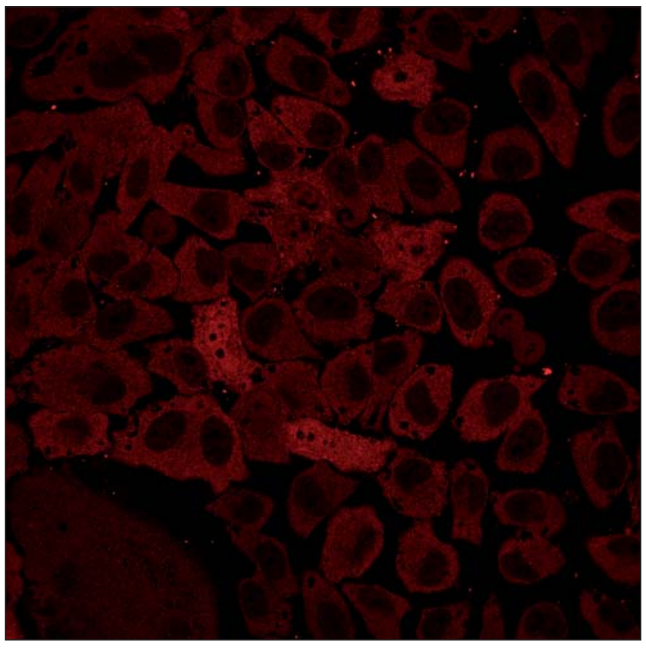

Fig. 2. H1299 cells were transfected with p53 and stained for p53 (green) and Hsp70 (red). In cells expressing p53, nuclear localization of Hsp70 is observed. Nuclei were counterstained with Hoechst 33342 (blue). (M. Olszewski)

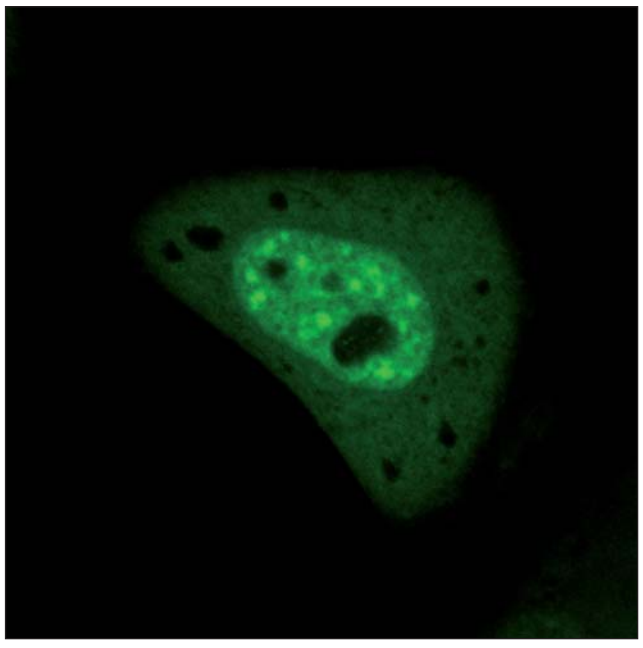

Fig. 3. H1299 cells were transfected with p53 and Hsp70-EYFP fusion protein and subjected to 42°C heatshock. Small nuclear aggregates visible in YFP (Hsp70) channel also contain p53. (M. Olszewski)

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ALZHEIMER'S DISEASE MUTATIONS AND γ-SECRETASE MEMBRANE PROTEASE

γ-Secretase is an integral membrane protease, which is a complex of four membrane proteins. Improper functioning of γ-secretase was found to be critical in the pathogenesis of Alzheimer's Disease. Despite numerous efforts, the structure of the protease as well as its proteolytic mechanism remains poorly understood.

We constructed a model of interactions between two proteins forming γ-secretase: APH-1 and presenilin. This interface is based on a highly conserved GxxxGxxxG motif in the APH-1 protein, and it can form a tight contact with a small-residue AxxxAxxxG motif in presenilin. We proposed and verified four binding modes based on similar structures involving GxxxG motifs in glycophorin and aquaporin. The resulting best model employs antiparallel orientations of interacting helices and is in agreement with the currently accepted topology of both proteins. All three hydrophobic regions (HRs) (8-10) in PS-1 CTF bear the GxxxG motifs. However, HR8 directly forms the catalytic site and HR10, containing the PAL region, is also recognized as being essential for the catalytic activity of γ-secretase. Furthermore, HR10 is not involved in the formation or stabilization of the γ-secretase complex. Taking into account the fact that HR8 and HR10 are involved in substrate processing, HR9 seems to be the most plausible region to form an interface for interaction with APH-1 (Fig.1).

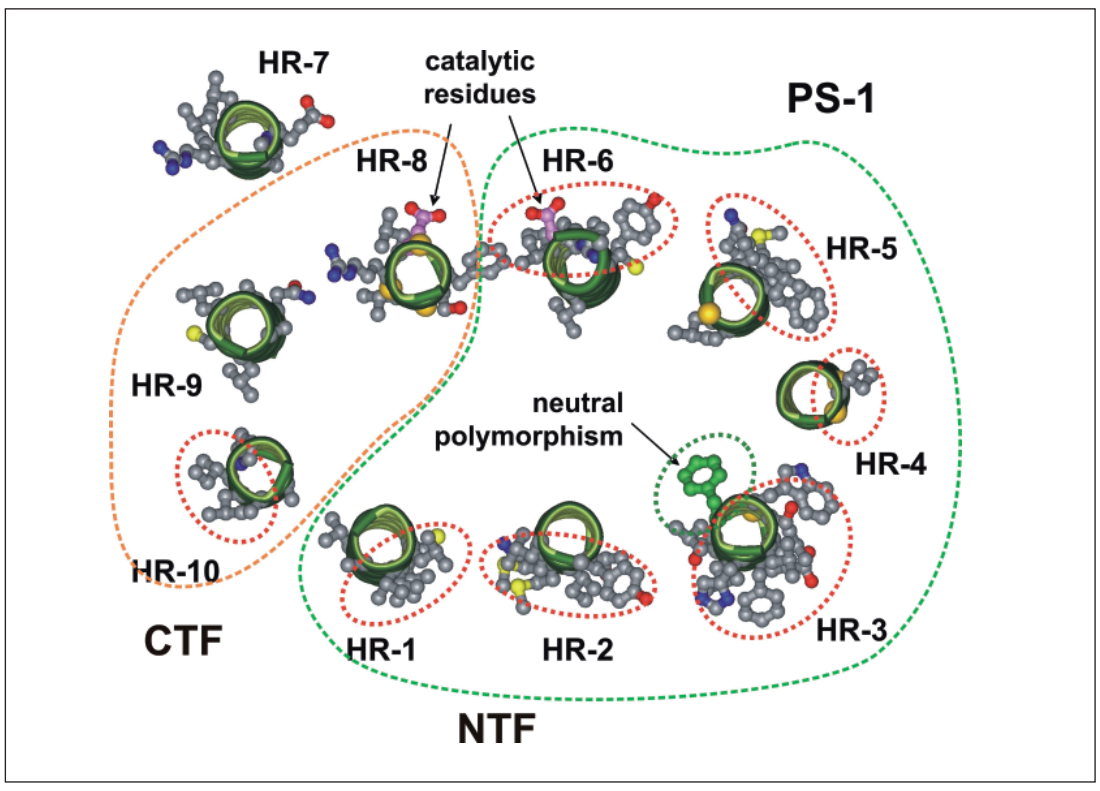

Fig. 1. The conceptual model of the membrane part of presenilin (PS-1) constructed based on the areas of PS-1 hydrophobic residues (HRs) containing Alzheimer's Disease mutation residues. N- and C-terminal fragments are marked by dashed lines. Dashed red ellipses indicate concentrations of AD mutations on particular faces of helices. Dashed green ellipse denotes region containing neutral polymorphism. (S. Filipek)

In antiparallel orientation the 409AxxxAxxxG417 motif located on the PS-1 HR9 helix can interact with an extended 118AxxxGxxxGxxxG130 motif found in APH-1 TM4. This is because of a much smaller helix-helix angle compared to the parallel models. Paradoxically this extension diminishes the contact surface, but also greatly improves the binding energy. This strongly suggests that the antiparallel models better approximate the interface. The framework of adjacent side chains is essential for creating stable helix interaction, so each case of (small)xxx(small) motif must be tested independently.

In the case of the PS-1 – APH-1 interface analyzed, there is a long network of small residues (Fig. 2) and, additionally, a hydrogen bond between the interacting helices cements them. Such an arrangement of residues in antiparallel model provides the lowest binding energy suggesting the most probable mode of interaction. This model can be used for further structural characterization of γ-secretase and its components.

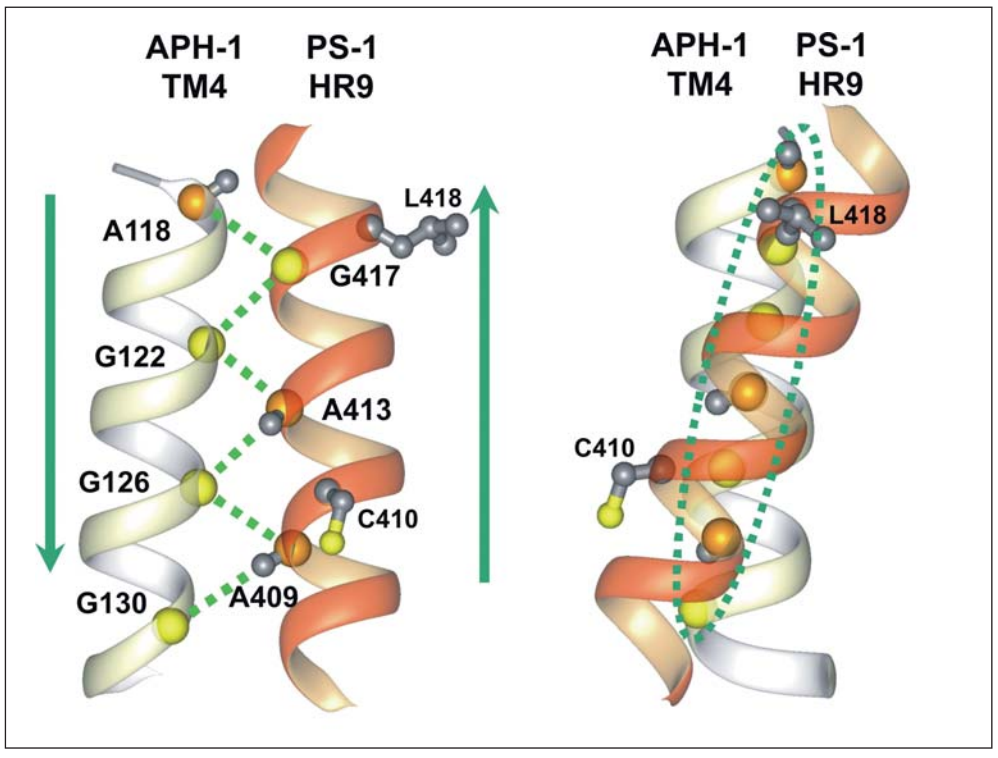

Fig. 2. The molecular model of the interface between APH-1 and PS-1 based on a double GxxxG motif. Cα of Gly and Ala forming the interface are shown as yellow and orange spheres, respectively. Green arrows indicate directions of helices. The elongated contact area in the interface is indicated by an ellipse on the right image. (S. Filipek)

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CRYSTALLOGRAPHIC STUDIES OF BIOLOGICALLY IMPORTANT PROTEINS

The MPI-CBG group at the International Institute of Molecular and Cell Biology in Warsaw has worked on peptidases and endonucleases.

Peptidases:

Protein peptidase inhibitors face a dilemma – on the one hand, they must block the active sites of their target proteins, but on the other hand, they can retain their potency only if they are not cleaved like substrates. Several strategies have evolved to solve the dilemma: some inhibitors span the active site cleft of proteases, but meander around the active site so that they never come dangerously close to the protease nucleophile. Others bind to the active site cleft, but in opposite orientation to the substrate. Still others form a covalent acyl-enzyme intermediate with the peptidase, but block the deacylation step. Paradoxically, some also bind like substrates, but are not cleaved. Why so? The classical answer has been that binding is so tight and the ends are held together so closely after cleavage that the back reaction (synthesis of the inhibitor from its fragments) dominates over the forward reaction (inhibitor cleavage). In serine proteases, this mode of inhibition is common (known as the Laskowski or standard mechanism), but prior to our work, nothing similar had been observed for cysteine protease inhibitors. Together with our co-workers in Kraków, Prof. Adam Dubin and Prof. Jan Potempa of Jagiellonian University, we have discovered that protease inhibitors known as staphostatins bind to cysteine proteases known as staphopains "almost" like substrates. Intriguingly, this crystallographically observed protease-inhibitor interaction is very reminiscent of the "ion-molecule" complex that was proposed as an intermediate in catalysis by Peter Kollman based on quantum chemical calculations many years prior to our experimental studies.

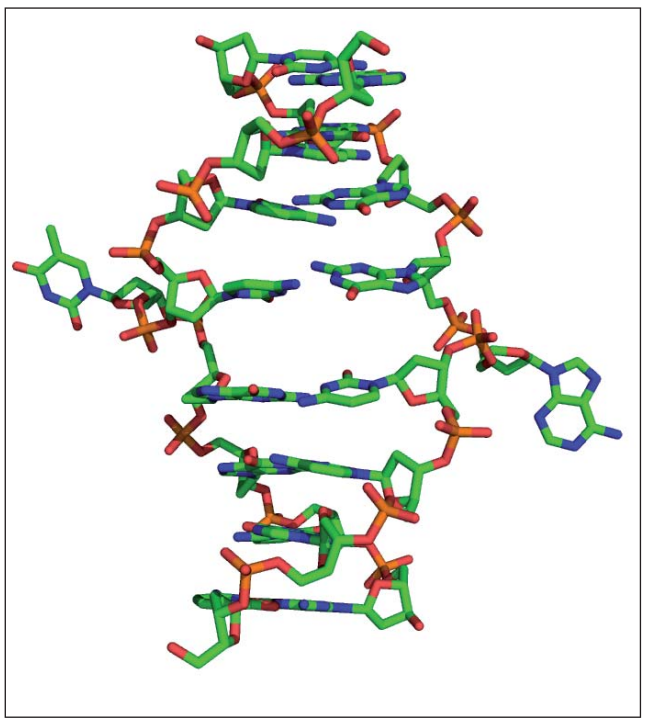

Fig.1. DNA base flipping by restriction endonuclease Ec118kI (M. Bochtlre)

Endonucleases – nucleotide flipping:

Nucleotide flipping, the extrusion of a base from the base stack in the helix into an extrahelical position, is a well-known process that may occur spontaneously, but is rare. Many enzymes promote this drastic distortion of DNA, usually because they need access to the base to excise it or to alter it chemically. In some cases, the flipping also provides access to a damaged estranged base in the stack. We have discovered the first instance of nucleotide flipping in restriction endonucleases. Flipping in this group of enzymes is unusual for several reasons: (a) It naturally occurs with intact base-pairs, and is not intended to detect or repair a lesion. (b) Nucleotides in both DNA strands are flipped, and the DNA is compressed. The other enzyme classes usually flip only one base and leave the estranged base in place to prevent DNA compression. (c) In some cases (Ecl18kI), flipping serves to "skip" a base pair in a pseudopalindromic target sequence. (d) In other cases (PspGI, EcoRII), it helps to distinguish A:T pairs from G:C pairs with a million-fold specificity.

Therefore our structures of Ecl18kI and PspGI highlight previously "unrecognized" uses of nucleotide flipping.

Endonucleases – links to the DNA repair machinery:

An emerging theme in restriction endonuclease biology is the link between cellular enzymes that play a "constructive" role in DNA repair and restriction endonucleases that play a "destructive" role in the defence against invading DNA. Using protein crystallography, we have shown that MvaI and BcnI resemble MutH more closely than any other structurally characterized restriction endonuclease. As the nickase MutH is a central player in DNA damage repair, our findings contribute to the growing understanding of links between DNA maintenance and destruction.

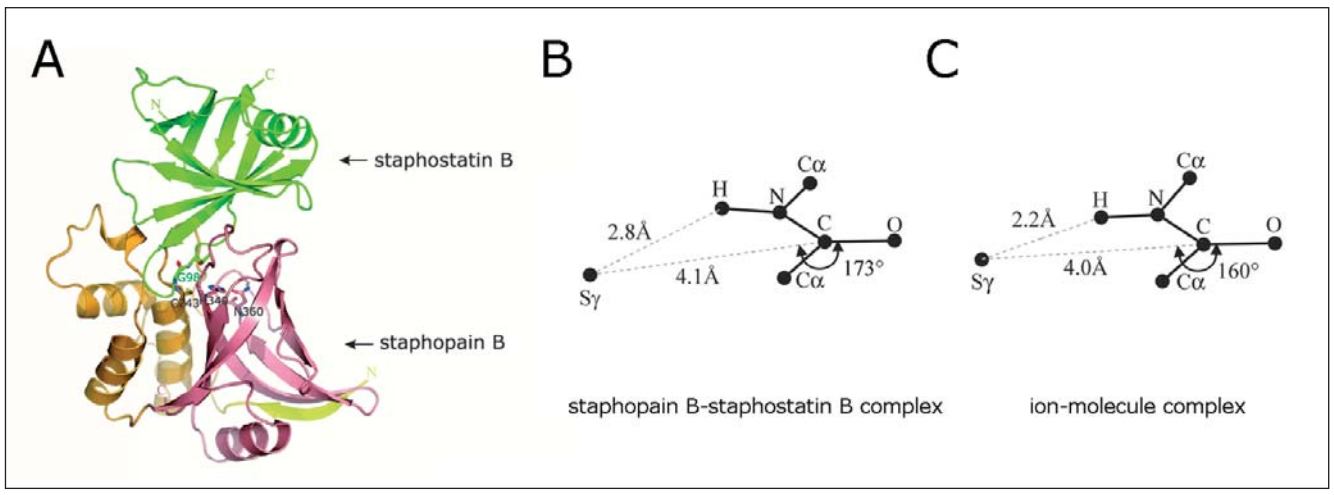

Fig. 2. Ribbon representation of staphopain B-staphostatin B structure (A), location of the active site nucleophile atom relative to the potentially reactive amide bond in the wild-type staphopain B-staphostatin B complex (B) and in the theoretically derived ion-molecule complex (C) (M. Bochtler)

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ESTIMATION OF GENETIC VARIABILITY OF THE EUROPEAN BISON USING BOVINESNP50 GENOTYPING BEADCHIP (ILLUMINA®)

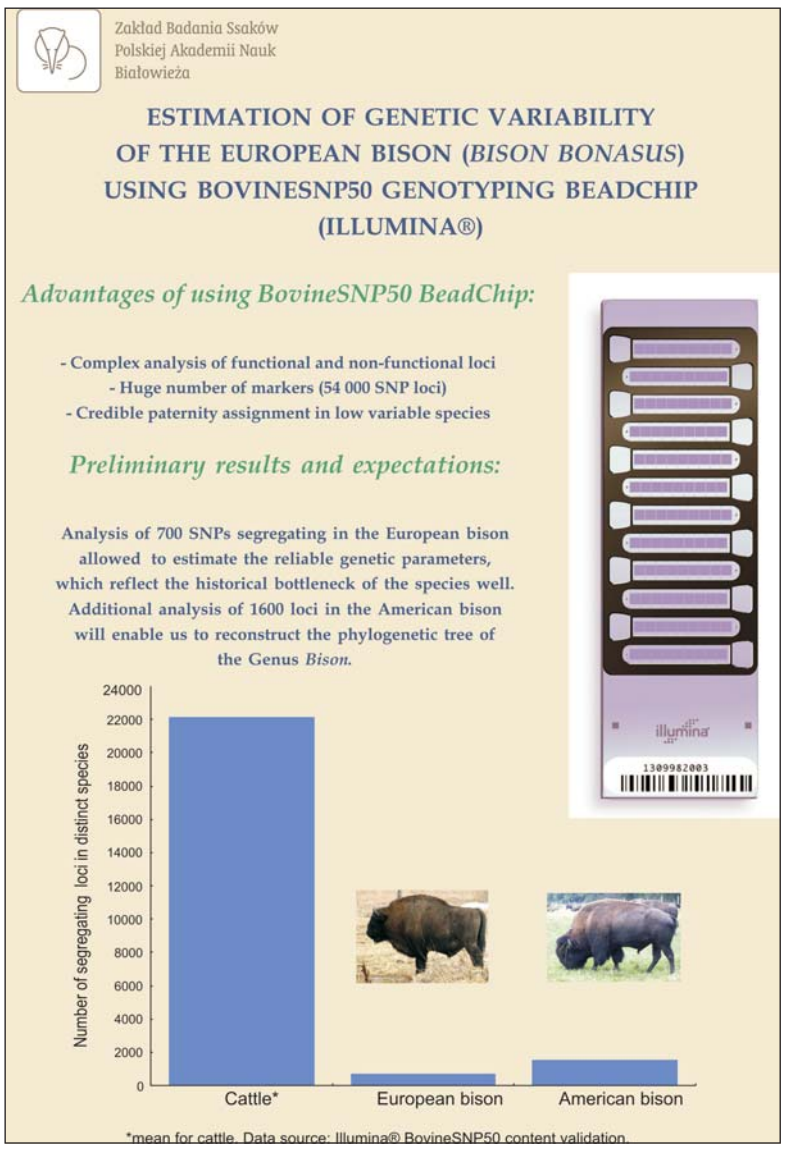
The Białowieża European bison (*Bison bonasus*, L.), Europe's largest mammal species, went through a severe bottleneck at the beginning of the 20th century. Only seven individuals founded the Lowland line of European bison and nearly 90% of the genes in the contemporary population derive from only two founders. The genetic diversity of the species is very low, threatening its survival. The European bison's viability, however, does not seem to be directly related to its level of heterozygosity and has more to do with complex intra- and inter-gene interaction which can only be investigated with quantitative genetic approaches. Given the fact that evolutionary response in quantitative traits cannot be predicted using molecular measures, an approach which investigates both neutral (non-coding) and non-neutral (coding) markers is necessary in which quantitative genetic analysis will play a central role in the assessment of extinction risk in conservation biology.

European bison bull (R. Kowalczyk)

European bison herd (R. Kowalczyk)

Attempting to correlate neutral and non-neutral variability is now possible by using a new and very promising tool in conservation genetics: single nucleotide polymorphisms (SNPs), which are the most abundant polymorphic genetic marker in most genomes. SNPs hold the potential to significantly expand our ability to survey both neutral (non-coding region) variation as well as genes under selection (coding region) in natural populations, providing broader genome coverage compared to mitochondrial DNA or microsatellites. The BovineSNP50 BeadChip (ILLUMINA®) offers 54 000 SNP loci uniformly covered with an average probe spacing of 51.5 kb, providing satisfying SNP density for robust genome-association studies in cattle.

The studies performed so far on 11 European bison samples have enabled reliable estimation of genetic parameters while subsequent association of additional samples of the American bison will provide a detailed genetic comparison between the two species and create a credible phylogenetic tree with other Bovids.



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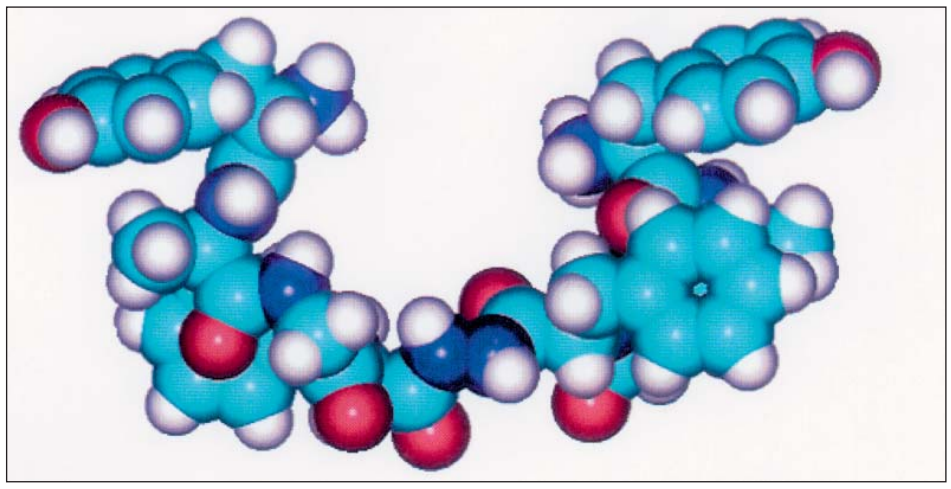
MULTITARGET NEUROPEPTIDE ANALOGUE BIPHALIN AS A NEW MEDICINE FOR CHRONIC PAIN TREATMENT

The prolonged life expectancy of the current population is a spectacular success of modern medicine. However, chronic pain that is associated with age related diseases or the result of surgical interventions poses a real medical challenge. Application of oral pills (morphine) or transdermal patches (fentanyl) with lipophylic analgesic drugs is the most common treatment for strong chronic pain. Penetrating into central nervous system, these compounds produce side effects (respiratory depression, constipation, tolerance, sedation, etc.) to such an extent that pain treatment is reduced by doctors or refused by patients. The development of new types of analgesics is therefore one of the urgent needs of contemporary medicine.

In response to these needs, an international consortium for the development of a new generation of analgesics has been created. The development of selective compounds interacting with one target receptor system is a traditional way of searching for new medicines. Unfortunately, in the case of the neuronal system, its strong plasticity has resulted in fast adaptation to medicines that induced tolerance and sometimes dependence to chronically used drugs. Several years ago we proposed to develop a new generation of analgesics based upon endogenous opioid peptides. This group of analgesics would be characterized by high spatial (i.e. compartmental) selectivity and affinities to the broad spectrum of receptor targets involved in nociceptive modulation. The distribution selectivity of opioid peptides is due to both susceptibility to enzymatic degradation, and their limited permeability through biological barriers including the blood-brain barrier. Therefore, opioid peptides applied at the site of their expected action could interact locally with a range of target nociceptive receptors, but in actual practice would not be able to interfere with distant systems. One opioid peptide analogue that fulfills the criteria of this new generation of analgesics is biphalin.

Biphalin is a dimeric peptide [(Tyr-D-Ala-Gly-Phe-NH–)$_2$] in which two enkephalin type pharmacophores are connected "head-to-head" by a hydrazide bridge. It has high affinity to all types of opioid receptors. Administered directly to the central nervous system, biphalin has been shown to be an analgesic over a thousand times more potent than morphine. When administered intravenously,



Biphalin structure (space filling model)

it expresses antinociceptive potency comparable to that of morphine, consistent with the limited permeability of biphalin through the blood-brain barrier. In addition to direct analgesic activity biphalin expresses several very promising adjunct effects, like antiproliferation of cancer cells and a synergic effect to standard multidrug AIDS treatment. Current activity of our research group is focusing on developing biphalin as a new analgesic drug.

Biphalin synthesis and application as an analgesic are covered by national (PL-0355470) and international (WO-014943) patents. For further development of biphalin as a new analgesic drug, a "spin-off" company has been created. The project has received a special grant covering the necessary preclinical toxicological and clinical phase I studies.

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