PROJECT SUMMARY

"Chip Technology for the Detection of Growth Hormone Abuse"

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Recombinant human growth hormone (rGH) has found wide-spread appliance in the sport community in the believe that it has ergogenic effect and the understanding that its (mis)use cannot be perceived by current analytical methodologies. Efforts to detect indirect evidences (biochemical markers) of rGH use are encouraging but never will be a definitive proof of the drug. Direct distinction of rGH from endogenously produced GH is difficult based only on the amino acid sequences, which appear to be identical. Additionally, the secretion of the endogenous hormone is a pulsatile event in nature, and that appears to retort differently depending on the kind of exercise performed. An absolute quantification as a single parameter for the detection of misuse is therefore unsustainable.

A consequence of the administration of rGH is the down regulation of the endogenous production. As the endogenous hormone consists of an array of molecular-weight "isoforms", due to splice-variants and other processes, the consumption of rGH, composed usually of only one of the possible "isoforms", is reflected by variations in the ratio between the different isoforms in the body fluids. An accurate and reliable quantification of this ratio should disclose the origin of the detected GH, as has been suggested by other researchers. This process can be implemented on a chip surface for rapid and high throughput screening by means of surface plasmon resonance (SPR). Additionally, SPR allows unambiguous characterisation of the binding properties of antibodies, both commercially available and generated by the partners in the project, directed against the different known GH "isoforms" . Simultaneously, the proposal includes the scrupulous analysis of the different recombinant human GH preparations, as well as the natural hormone, by state-of-the-art mass spectrometric (MS) equipment, aims at disclosing possible structural differences arising from post-translational modifications of the amino acids and may result in the production of novel antibodies with conclusive discriminatory capacity. Combinations of different antibodies will be screened, in a single analytical setting, to establish the optimal combination to quantify "isoform" ratios.

~ The project will deliver a test system available for rapid screening rGH administration at the laboratory. MS characterisation of GH "isoforms". (Expected before summer 2004)

Concurrently, generated peptide-fingerprints will serve as reference data for the positive identification of (r)GH. The MS characterisation will be implemented/ applied to samples derived from SPR analyses. The semi-hyphenation of both techniques provides an extremely powerful combination for the detection,

quantification and unambiguous structural characterisation of human growth hormone, directly from complex biological matrices such as urine or blood.

~ The project will deliver the specifications for an "on site" screening device and the laboratory mass spectrometric confirmation, directly from the sensor chip.

Finally, the approach will be validated using the large number of relevant samples, available from the partners and generated during a clinical study. Additionally, and given the interest in detecting the GH related peptide IGF-1 (potential doping either by recombinant product or gene transfer), preliminary data regarding the feasibility of the same approach for IGF-1 detection will be studied.

> The project will deliver the validation of the methodology proposed and the exploration of its use for other doping growth factors.

Results and Conclusions

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Growth hormone comprises a multifarious family of different splice variants, full-length peptides, proteolytic fragments, homo-, di- and oligomers, and hormone-binding protein complexes. It has been shown that exogenous administration of recombinant 22 kDa GH alters the ratio of the normal isoform balance and that abuse could be detected possibly through isoform-ratio monitoring. The proposal focussed on the 22 and 20 kDa isoforms and originated from the idea that an isoform ratio quantification could be done through either ELISA or Surface Plasmon Resonance (SPR). Whereas the former is a well-established technique that provides information on an "end-point" situation, the latter technique enables both qualitative and quantitative monitoring of multiple antibody-antigen interactions in parallel, real time, under near-physiological conditions and without the need for labelling or a secondary antibody. With this approach several specific and non-specific anti-GH antibodies have been characterised in terms of thermodynamic constants such as association rate and dissociation rate constants, specificity and cross reactivity, surface properties, sensitivity, linear range and so forth. Knowledge of these aspects has proven of utmost importance when different isoforms of the same molecule are to be quantified employing distinct antibodies. As such, it was observed that the anti-20 kDa antibody has inferior surface properties with respect to the anti-22 kDa antibody and that this can be partially compensated for using more surface-bound antibody. Also, another non-specific antibody was shown to display different dissociation rate constants for both isoforms for which a quantification of isoforms based on such an antibody would render different ratios as a function of the number of washes or the total elapsed time between interaction and measurement.

In short, the results have shown that it is indeed possible to perform isoform ratio measurements by means of SPR but the limitations in the instrumental design and the low capacity of the surface (approximately 15 ng or 100 fmol), compromises the sensibility and impedes an eminent implementation. Substantial efforts have also been invested, throughout the course of the project, in the sample processing (both for urine and plasma matrices) in order to bring concentrations within the range of the SPR technology as well as in the generation of well defined GH isoforms for the characterisation of the antibodies. Within this context a synthetic 5 kDa has been produced as well as proteolytic 5 kDa and 17 kDa isoforms (recombinant 20 kDa was already produced in Japan and recombinant 22 kDa material is available from different pharmaceutical companies). Furthermore, monoclonal antibodies towards the distinct isoforms have been generated and characterised by means of SPR, or are in the process of being produced. With all information a refined, highly sensitive, differential ELISA system, monitoring the 22 and 20 kDa isoforms, has been validated with limit of detection (LOD) of about 5 pg/ml for which plasma samples can be monitored directly and urine samples with minor processing. This ELISA has been screened with a large number of ordinary samples and is currently in the phase of beta testing employing samples generated through a clinical study with rhGH. Further developments lie in the marketing of the ELISA as well as the advanced transfer of this technology to a higher throughput platform such as Luminex using flowcytometry as basic methodology