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THE OVERLOOKED DIFFERENCE:
Structural basis for a mass spectrometry-based dopingtest for recombinant erythropoietins

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Extended abstract

During the past couple of years several strategies have been developed in order to detect doping with recombinant erythropoietins (rhEpos), namely direct (IEF-PAGE in carrier ampholytes [1], SDS-PAGE [2], 2D-PAGE, Sarcosyl-PAGE [3], ELISA [4], MAIIA (membrane-assisted isoform immunoassay; [5])) and indirect methods (based on blood parameters and statistical models [6]). Two matrices have to be used – urine and serum/plasma – the latter for enabling the detection of the abuse of PEGylated rhEpos (e.g. MIRCERA). Aside from differences in the isoform profile on IEF-PAGE and in the molecular mass on SDS-PAGE [7] little is known about the detailed glycan structures of endogenous human erythropoietin(s). This is manly due to the extreme low abundance of this regulatory protein in both blood and urine (typically in a concentration of low pg/mL, i.e. amol/mL) and the lack of highly purified reference materials for the characterization of human urinary and serum/plasma Epo (uhEpo, shEpo). A breakthrough in the isolation of uhEpo was achieved by Miyake et al. in 1977 [8] who used 2550 liters of urine of anemic patients (who excreted uhEpo in an about one thousand-fold higher concentration than normal individuals) in order to obtain milligram amounts of this cytokine. The following three decades (i.e. 33 years) brought only a handful of publications dealing with the glycan structure of uhEpo [9, 10] and shEpo [7], and on the other hand numerous detailed (and, by the way, mostly repetitive) publications on the structure of rhEpos [11].

However, during all these 33 years an obvious and profound structural difference between endogenous (uhEpo, shEpo) and recombinant erythropoietins was overlooked. By sequentially removing the monosaccharides, which are known to compose the N-glycans and part of the O-glycan of Epo, with a series of exoglycosidases (so called “exoglycosidase array sequencing”)

the action of N-acetyl-β-D-glucosaminidase was partly blocked by uhEpo but not by rhEpos. In consequence, the subsequent cleavage with α-mannosidase was also blocked by uhEpo but not rhEpo – thus leading to a significant decrease in the apparent molecular mass of rhEpo in comparison to the mass decrease of uhEpo (Figure 1).

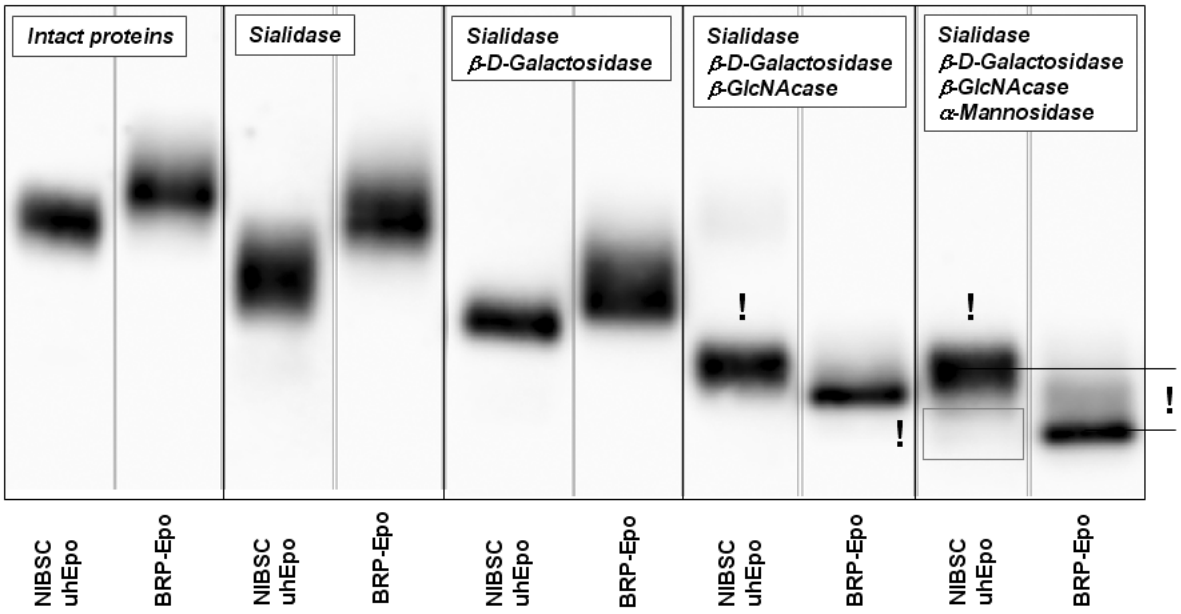

Figure 1: Exoglycosidase array sequencing of uhEpo (NIBSC standard) and rhEpo (BRP-Epo standard). Only uhEpo partly blocks the action of β-GlcNAcase but not rhEpo. The subsequent cleavage of the remaining N-glycan structures with α-mannosidase is feasible for rhEpo but not (i.e. only to a very limited extent) for uhEpo.

Over 30 recombinant epoetins (including many biosimilars) were tested and all of them showed the same behaviour, regardless of the cell line used for their production (CHO, BHK, HT-1080). The same blocking-behaviour was demonstrated for shEpo – indicating a close structural relationship between shEpo and uhEpo. Due to this profound structural difference a discrimination of all recombinant Epo-pharmaceuticals from human endogenous Epo was possible within a single molecular mass-based experiment (SDS-PAGE). An explanation for the altered behaviour of endogenous Epo might be the presence of a bisecting N-acetylglucosamine sugar residue, since the expression of glycosyltransferases (GlcNAcT) is tissue specific and CHO- and BHK-cells do not express GlcNAcT-III. GlcNAcT-III is highly expressed in human kidney but not in human liver cells.

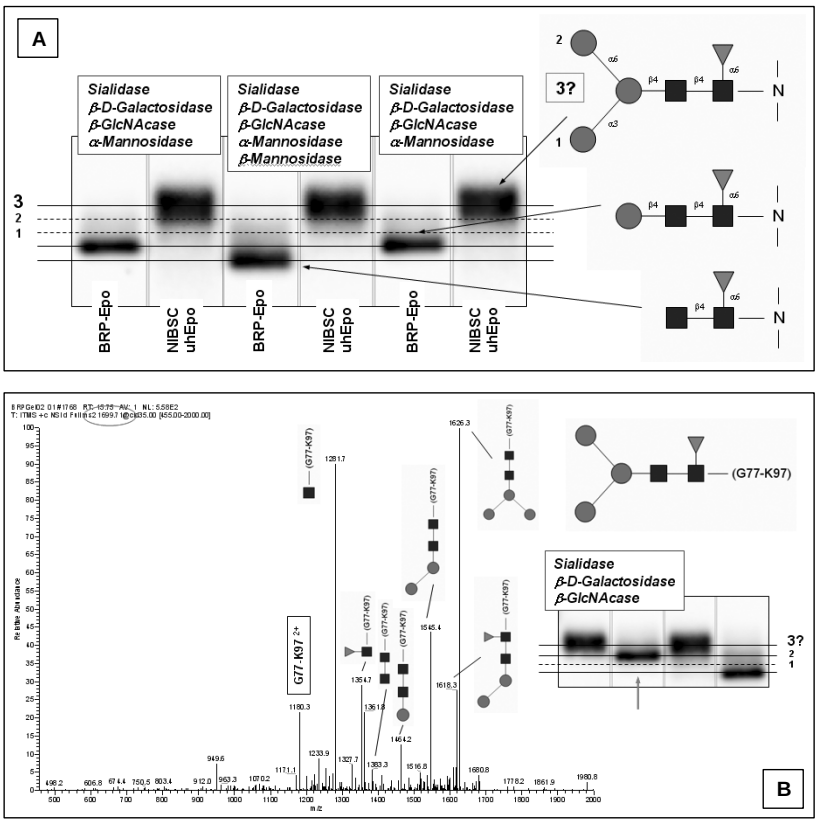

Figure 2: Mass spectrometric verification of the N-glycan structures on rhEpo after cleavage with 3 and 4 exoglycosidases. (A) Annotated Western blot of exoglycosidase-digested uhEpo and rhEpo, (B) mass spectrum of the 3 exoglycosidase-trimmed tryptic N-83 glycopeptide (77GQALLVNSSQPWEPLQLHVDK97).

Conclusions: (1) the sequential cleavage with 4 or 5 exoglycosidases generated N-glycan marker-structures, which allowed a simple differentiation between (all) rhEpos and uhEpo/shEpo; (2) these structures might be the basis for a targeted mass spectrometric test for rhEpo doping; (3) in order to verify the presence of N-acetyl-β-D-hexosaminidase blocking sugars on uhEpo/shEpo a detailed mass spectrometric study on endogenous Epo is overdue.

Note: For additional details please refer to the forthcoming full article (same author).

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