

Q-NMR-Based Metabonomics of Blood Samples

Cyclosporine A (CsA) is a potent calcineurin inhibitor used in transplantation medicine. It is potentially nephrotoxic. The use of CsA in combination with other immunosuppressants, such as sirolimus (SRL) or everolimus (RAD), has been reported to produce a beneficial synergistic effect. In this study, blood samples were collected from rats treated with CsA (10 mg/kg), CsA(10 mg/kg) + SRL(3 mg/kg), and CsA(10 mg/kg) + RAD (3mg/kg). Whole blood samples were collected and processed by dual chloroform/methanol extraction to yield water- and lipid-soluble extracts. The extracts were analyzed by Q-NMR to predict the metabolic toxicity, and to identify and quantify metabolic biomarkers. A 600 MHz NMR spectrometer was used and the proton NMR spectra obtained by coaddition of 40 transients, using a relaxation delay of 12 s and a tip angle of 90°. The water resonance was suppressed by selective saturation. A solution of trimethylsilylpropionic acid- d_4 (TMSP- d_4 , 0.00 ppm) was placed in a capillary and inserted into the NMR tube for use as an external standard. All spectra were normalized to the intensity of the TMSP- d_4 singlet resonance.

To obtain accurate and meaningful information, NMR spectral data must be carefully processed prior to attempting statistical analysis of the results. In this experiment, Fourier transformation, phase correction and baseline correction were performed. For multivariate statistical analysis, all spectra were normalized to the TSP- d_4 intensity and the full spectrum was bucketed into 0.04 ppm intervals, except the regions containing the solvent resonances of water and methanol which were excluded from the statistical analysis. Principal component analysis (PCA) was performed using the AMIX 3.1 software to classify the NMR spectra obtained from animals subjected to different experimental treatments. For metabolite quantification, each ^1H peak of identified metabolites was integrated. Absolute concentrations of the identified metabolites were calculated using the equation shown below:

$$C_x = \frac{\frac{I_x}{N_x} \times C_{TMSP}}{\frac{I_{TMSP}}{N_{TMSP}}} \times \frac{V}{M}$$

where C_x = metabolite concentration; I_x = integral of the metabolite ^1H NMR resonance; N_x = number of protons giving rise to the metabolite ^1H peak (from CH, CH₂, CH₃, etc); C_{TMSP} = TMSP concentration; I_{TMSP} = integral of TMSP ^1H resonance at 0 ppm; N_{TMSP} = 9 because this resonance is produced by the 9 equivalent protons of the 3 methyl groups; V = volume of the extract; and M = volume of the blood sample.

In the results obtained by PCA from the PC1 vs PC2 scores plot, spectral results for all 5 placebo-treated control animals clustered together and were overlapped with the samples for the CsA+RAD treated animals. The results for the CsA treated animals were well-separated from the control animals. The spectral results that were most different from the controls were those of the CSA+SRL treated animals, which clustered as their own group distinct from the CsA-only treated animals. The PCA loadings plot indicated that the intensities of the following metabolites differed among the CsA+SRL, CsA, and placebo groups: hydroxybutyrate, lactate, total glutathione, creatine + creatinine, trimethylamine-N-oxide (TMAO), and glucose. The only metabolite that increased in all 3 treatment groups was cholesterol. QNMR measurements of individual metabolite levels showed that CsA administration significantly increased the blood concentrations of glucose, hydroxybutyrate and creatine + creatinine. However the levels of glutathione dropped in both CsA and CsA+SRL treated animals. The blood levels of these

metabolites were not significantly different for the CsA+NAD treated animals and the placebo-treated controls.

The increase in blood glucose and hydroxybutyrate confirmed the ability of CS to induce hyperglycemia and hyperketonemia. The decreased levels of glutathione were thought to be related to CS-induced oxidative stress. The increased concentrations of metabolites such as creatine and creatinine could reflect decreased renal clearance of these substances. While co-administration with SRL enhanced the metabolic changes indicative of toxicity, combination treatment with RAD partially alleviated these effects. This example illustrates the utility of metabolic profiling by Q-NMR and the need to monitor the toxicodynamic effects of immunosuppressant combinations.

Reference

Serkova, N. J. and Christians, U. *Ther. Drug. Monit.* **2005**, *27*, 733-737