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LC-MS analysis of free and esterified fatty acids.

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An abbreviated version of this protocol was published in The Journal of Clinical Investigation in Apr 2022

IL-9/STAT3/fatty acid oxidation–mediated lipid peroxidation contributes to Tc9 cell longevity and enhanced antitumor activity

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Detailed protocol

Fatty Acid Extraction

Internal standard mixture consisted of 12.5 µg/mL of (1, 2, 3, 4, 5, 6-$^{13}C_6$) docosanoic acid and 50 µg/mL ^{13}C-labeled myristic, palmitoleic, palmitic, margaric, linoleic, oleic, elaidic, and stearic acid in ethanol (Cambridge Isotope Laboratories, Tewksbury, MA, USA). To sample containing tubes, pre-washed with methanol, 32 µL of internal standard mixture, 1 µL of 10 mM butylated hydroxytoluene in methanol and 1.5 mL of ice-cold methanol was added. Samples were homogenized using a liquid nitrogen cooled Precellys Evolution bead mill homogenizer, vortexed 10 min at room temperature, allowed to sit on ice 10 min then centrifuged at 17,000 (x g), at 4 °C, for 10 min. The supernatants were then transferred to 2 mL vials with Teflon caps and dried using nitrogen gas.

Fatty Acid Picolylamide Derivatization

Extracted free fatty acid acids were converted to acyl chloride intermediates by treatment with 300 µL of 2 M oxalyl chloride in dichloromethane, at 65 °C, for 5 min. The solutions were then dried using nitrogen gas. Dried samples were further derivatized by adding 225 µL of 1% (v/v) 3-picolylamine in acetonitrile. The reaction was incubated at room temperature for 5 min. Finally, the solutions were dried using nitrogen gas.

Reverse Phase Liquid Chromatography

Dried derivatization products were reconstituted in 100 µL ethanol, transferred to glass auto-sampler vial inserts and centrifuged at 4,000 *g*, at 4 °C, for 3 min. Supernatants were next transferred to fresh vial inserts for analysis. The injection volume was 10 µL. Mobile phase A (MPA) was water containing 0.1 % formic acid, and mobile phase B (MPB) was acetonitrile containing 0.1 % formic acid. The chromatographic method included a Thermo Fisher Scientific Accucore C30 column (2.6 µm, 150 x 2.1 mm) maintained at 15 °C, autosampler tray chilling at 8 °C, a mobile phase flowrate of 0.500 mL/min, and a gradient elution program as follows: 0-5 min, 5% MPB; 5-40 min, 5-95% MPB; 40-85 min, 95% MPB; 85-85.1, 95-5% MPB; 85.1-90 min, 5% MPB.

Tandem Mass Spectrometry

A Thermo Fisher Scientific Orbitrap Fusion Tribrid mass spectrometer with heated electrospray ionization source was operated in data dependent acquisition mode with a scan range of 150 - 550 *m/z*. Orbitrap resolutions of 120,000 (FWHM) and 30,000 for MS1 and MS2 were used, respectively. The instrument was operated in positive ionization mode with a spray voltage of 3,600 V, and vaporizer and capillary temperatures set at 350 and 325 °C, respectively. The sheath, auxiliary and sweep gas pressures were 50, 10, and 1 (arbitrary units), respectively. Ions were fragmented using assisted HCD with stepped collision energies of 25, 30, and 35%.

Fatty Acid Extraction

For free fatty acid detection, samples were homogenized using a liquid nitrogen cooled Precellys Evolution bead mill homogenizer and mixed with internal standard mixture in ice-cold methanol. Extracted free fatty acid acids were converted to acyl chloride intermediates and further derivatized. Dried derivatization products were reconstituted in 100 µL ethanol, transferred to glass auto-sampler vial inserts and supernatants were next transferred to fresh vial inserts for analysis. Mobile phase A (MPA) was water containing 0.1 % formic acid, and mobile phase B (MPB) was acetonitrile containing 0.1 % formic acid. The chromatographic method included a Thermo Fisher Scientific Accucore C30 column (2.6 µm, 150 x 2.1 mm) maintained at 15 °C, autosampler tray chilling at 8 °C, a mobile phase flowrate of 0.500 mL/min, and a gradient elution program as follows: 0-5 min, 5% MPB; 5-40 min, 5-95% MPB; 40-85 min, 95% MPB; 85-85.1, 95-5% MPB; 85.1-90 min, 5% MPB. A Thermo Fisher Scientific Orbitrap Fusion Tribrid mass spectrometer with heated electrospray ionization source was operated in data dependent acquisition mode with a scan range of 150 - 550 *m/z*. Orbitrap resolutions of 120,000 (FWHM) and 30,000 for MS1 and MS2 were used, respectively. The instrument was operated in positive ionization mode with a spray voltage of 3,600 V, and vaporizer and capillary temperatures set at 350 and 325 °C, respectively. The sheath, auxiliary and sweep gas pressures were 50, 10, and 1 (arbitrary units), respectively. Ions were fragmented using assisted HCD with stepped collision energies of 25, 30, and 35%

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1. Yi, Q. and Xiao, L. (2022). LC-MS analysis of free and esterified fatty acids.. Bio-protocol Preprint. bio-protocol.org/prep1738.
2. Xiao, L., Ma, X., Ye, L., Su, P., Xiong, W., Bi, E., Wang, Q., Xian, M., Yang, M., Qian, J. and Yi, Q.(2022). IL-9/STAT3/fatty acid oxidation–mediated lipid peroxidation contributes to Tc9 cell longevity and enhanced antitumor activity. The Journal of Clinical Investigation 132(7). DOI: 10.1172/JCI153247