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A Novel Acrylonitrile Compound as Inhibitor of NADPH Oxidase 2

The NADPH oxidase system, NOX2, is responsible for reactive oxygen species (ROS) production in neutrophils and has been recognized as a key mediator in inflammatory disorders and several cardiovascular pathologies (1). Nevertheless, there is a lack of specific inhibitors of NOX2, due to the complexity of the system and the existence of several NOX isoforms (2).

For this purpose, we tested the hypothesis that a novel acrylonitrile compound may be a NOX2 inhibitor. We synthesized a new acrylonitrile: (2*E*)-2-(1*H*-indol-3-ylcarbonyl)-3-(5-phenylisoxazol-3-yl)acrylonitrile, C3f). C3f was synthesized using the microwave-assisted Knoevenagel condensation (3). We tested the capacity of C3f to inhibit NOX2 derived ROS production upon stimulation in human neutrophils.
HL-60 cells (human, promyeloblast, ATCC-No.CCL 240) were differentiated to neutrophils (DMSO 1.3 % for 7 days) and stimulated with phorbol 12-myristate 13-acetate (PMA 0.8 µmol/L). ROS production was assessed monitoring the fluorescence of 2',7'-dichlorofluorescein (DCF) in an automated fluorescence plate reader. In this assay, C3f presented a concentration-dependent inhibition of PMA-stimulated ROS production, with an IC_{50} 1.098 µmol/L.

Using Western blot analysis of $p47^{phox}$, a cytosolic subunit of NOX that translocates to the membrane upon activation, we evaluated $p47^{phox}$ translocation stimulated by PMA. Cf3, 50 µmol/L, significantly reduced the translocation of $p47^{phox}$ (ratio membrane/cytosol of $p47^{phox}$ 4.57 ± 0.56 control vs. 0.88 ± 0.25 C3f, $p<0.0002$, n= 3.

A second series of experiments was performed, using HL-60 membranes, in which we used cytochrome *c* reduction as an alternative measure of superoxide production in a cell-free system. In this condition, C3f minimally reduced the production of superoxide:108.6 ± 23, 78.5 ± 15 and 67.6 ± 14 % of control at 0.1, 1 and 10 µmol/L, n=3, $p>0.05$

Next, we evaluated Cf3 in cardiac myocytes from MDX mice, a model of dystrophic cardiomyopathy, reported to overexpress NOX2 (4). Cell were isolated using collagenase digestion, and superoxide production was assessed using DCF (5 µmol/L). Treating MDX myocytes with Cf3 1 µmol/L, 30 min, reduced ROS production from 83.9 ± 5.7 fluorescence units (intensity/pixels) in control cells (n=86, from 2 hearts) to 26.6 ± 1.4 in Cf3-treated cardiomyocytes, n= 71 (from 2 hearts), $p<0.0001$.

Additionally, C3f showed minimal cytotoxicity evaluated as MTT assay, only at 72 hrs of exposition, and did not show ROS-scavenging properties, evaluated as 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay (14, 13 and 18% of DPPH decoloration at 30, 50 and 150 µmol/L of C3f).

These results suggest that C3f, an acrylonitrile compound, may be a new NOX2 inhibitor, by inhibiting the activation of the NOX2 complex, devoid of radical scavenging properties and cytotoxicity.

(1) Wingler K et al (2011) *Br J Pharmacol* **164**: 866-883

(2) Wind S et al (2010) *Br J Pharmacol* **161**: 885-898

(3) De la Torre (2015) *JTICE, in press.*

(4) Gonzalez DR et al (2014) *Am J Physiol Heart Circ Physiol* **307**: H710–H721