Development of an in vitro model to test new putative Pannexin-1 inhibitors against neuropathic pain.

1)Trallori E. 2)Di Cesare Mannelli L. 3)Maresca M. 4)Tenci B. 5)Lucarini E. 6)Micheli L. 7)Awada K. 8)Crocetti L. 9)Giovannoni MP. 10)Ghelardini C.

University of Florence

Neuropathic pain arises from central or peripheral nervous system damage: not only it is caused by mechanical damage, but it ensues several diseases, like herpes zoster, hypoxia, stroke and multiple sclerosis, and it is also a detrimental side effect of chemotherapeutic treatment. Neuropathy is currently treated with opioids, sometimes assisted by anticonvulsants or antidepressants, but with low efficacy. There is the urgent need for a new drug target, which could lead to the development of an effective therapy against this pathology.

Pannexin-1 (Panx-1) is an integral protein, an hexameric channel spanning the plasma membrane of astrocytes, neurones, endothelial cells of the blood-brain barrier (BBB) and other non-nervous cell types (erithrocytes, macrophages, T-lymphocytes, etc.). Panx-1 channel is permeable to glutamate (Glu), ATP, potassium ions, calcium ions and other solutes, up to 900 Da molecular weight. It is mostly activated in pathological events: by extracellular ATP via the purinergic receptor P2X7, by a pro-apoptotic caspase3-mediated cleavage at C-terminus tail, by hypoxia, mechanical stress and high external potassium concentration.

In our recent study on oxaliplatin-induced neuropathy in rats, we showed a clear role for Panx-1 in the development of the chronic pain: Panx-1 exacerbates the Glu-excitotoxicity, initially released only by P2X7 which, when activated by ATP, recruites Panx-1 to release further ATP and Glu, exaggerating the pain signal. Panx-1 specific inhibitors reduced Glu release and were able to revert hyperalgesia and allodynia in neuropathic rats. Therefore blockage of this channel activity could be a valid alternative to current treatments.

The aim of this study is to set up an in vitro model on which testing new putative Panx-1 inhibitors.

First we produced a stock of Panx-1 overexpressing HEK293 cells: they were stably transfected with 3 µg plasmid encoding EGFP-tagged Panx-1 (PEM10) and maintained in selection for 9 days with gentamycin G418, as the plasmid contains gentamycin resistance gene. The Panx-1 overexpressing clones were assessed for the presence of the protein via FACS analysis, immunohistochemistry assay with Panx-1 antibody and via fluorescence microscope observation. Protein functionality was verified by dye uptake assays and ATP release assays.

Panx-1 was stimulated with an apoptotic stimulus to induce a caspase 3-mediated cleavage of the C-terminus, thus irreversibly activating the protein. Transfected HEK293 cells were incubated 1 h with 0.03 U/ml and 0.1 U/ml Glucose Oxidase (GOX)-containing medium, thus producing a noxious oxidative stress which could trigger apoptotic cascade and caspase 3 activation. Following incubation, the medium was removed and cells were incubated with a 20 µM Ethidium Bromide

solution for 15 minutes, to allow dye entry in the cells. The cells were then fixed in 4% paraformaldehyde and stained with DAPI.

Incubation with both concentrations of enzyme induced opening of pannexin channel and, therefore, entry of the dye in the nuclei, as observed at confocal microscope by merging EtBr fluorescence with DAPI staining.

ATP release assays were performed on the medium where cells were incubated with GOX and the results confirmed those obtained from dye uptake assay.

In the near future this protocol will be used to test putative Panx-1 inhibitor compounds, that will eventually be assessed for their efficacy in vivo against oxaliplatin-induced neuropathic pain.