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## Functional proteomics ties calreticulin with the Anti-Angiogenic Properties of the Pyrazolyl-Urea Gege-3

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In the last twenty years, 5-pyrazolyl-ureas have been largely investigated for their poly-pharmacological outline. In this scenario, ethyl 1-(2-hydroxypentyl)-5-(3-(3-(trifluoromethyl)-phenyl)-ureido)-1H-pyrazole-4-carboxylate (i.e., GeGe-3) emerged as a promising anti-angiogenic compound, inhibiting Human Umbilical Vein Endothelial cells (HUVECs) proliferation and endothelial tube formation, and blocking angiogenesis in mice and tumor growth in transplanted subcutaneous Lewis Lung Carcinomas1. Regrettably, although different primary targets implicated in cell division and/or calcium homeostasis have been hypothesized for this compound, all the performed tests gave negative results. Thus, to link GeGe-3 anti-angiogenic potential to a suitable protein partner, the molecule interactome has been inspected in HUVECs, through a label-free functional proteomics platform2,3 comprising Drug Affinity Responsive Target Stability (DARTS)4 and targeted Limited Proteolysis-Multiple Reaction Monitoring mass spectrometry (t-LiP-MRM)5. These techniques share the principle that, interacting with a molecule, a protein undergoes conformational changes that result in its altered sensitivity to limited proteolysis, when performed in native conditions. Thus, in a first step, pairing DARTS with high-resolution mass spectrometry allowed the identification of GeGe-3 most reliable interacting protein, calreticulin, as later on validated by Western Blotting. Subsequently t-LiP-MRM served the purpose of pinpointing calreticulin regions directly or distally involved in the interaction with the compound. Calreticulin is a major Ca2+ binding protein involved in intracellular Ca2+ homeostasis and uptake/release within the endoplasmic reticulum and mitochondria, cells adhesion, migration, proliferation, differentiation, and apoptosis, as well as in cell-cell interactions.Thus, to shed light on the biological consequences of GeGe-3 interaction with calreticulin, in cell assays have been performed. The obtained results disclosed GeGe-3 potential anti-angiogenic mechanism: as demonstrated by cytofluorimetry, the molecule alters Ca2+ intracellular shift in HUVECs and, as highlighted through confocal microscopy, induces F-actin to acquire a cortical localization, totally in line with cells showing a less motile phenotype.

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