UNRAVELING THE MECHANISM OF ACTION OF THE ANTIPROLIFERATIVE EFFECT OF FUROXANS IN SMOOTH MUSCLE CELLS: A PROTEOMIC APPROACH

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Atherogenesis is a multifactorial process characterized by oxidation, altered nitric oxide (NO) bioavailability and increased smooth muscle cell (SMC) proliferation in vascular intima. To find innovative antiatherosclerotic approaches, we studied a series of furoxan derivatives differently substituted at the 3- and 4- positions of the heteroring. These compounds are able to release NO in physiological conditions in the presence of thiol cofactors (free thiols or cysteine residues of proteins). Broadly speaking, the presence in the ring of electron withdrawing substituents, in particular at the 3-position, facilitates the nucleophilic attack of thiols at the ring and consequently the formation of the tetrahedric intermediate on the way of NO release. This is in keeping with the potency of these compounds to relax rat aorta strips pre-contracted with nor-epinephrine. The involvement of NO was evident by the co-administration of ODQ, a well-known inhibitor of soluble guanilate ciclase, which abolished the vasodilating effect.

On this basis we evaluated the effect of 3-Phenyl, 4-R furoxans and the corresponding isomers 4-Phenyl, 3-R furoxans (groups in 3 and 4 are interchanged) in inhibiting cell proliferation by cell counting and labelled thymidine incorporation into DNA of synchronized rat SMC.

These molecules, albeit with different potency, were able to inhibit SMC proliferation due to a specific cell-cycle inhibitory effect in G1/S phase. 4-Phenyl, 3-R furoxans were definitively more potent inhibitor of SMC proliferation than the corresponding 3-Phenyl, 4-R furoxans, whereas the related furazans (structurally very close to furoxans but unable to release NO), were not effective, supporting the fact that the opening of the ring is essential for SMC growth-inhibition. Interestingly, in the 4-Phenyl, 3-R furoxan series the inhibitory potency on SMC proliferation paralleled the vasodilation potency, in agreement with the susceptibility of the 3-position to the nucleophilic attack of thiol groups.

Since our experimental evidence suggested that the antiproliferative properties are not mediated by NO, through the cGMP- or the polyamines pathway, to exclude a different NO-mediated mechanism, we evaluated whether a co-treatment with classical NO-donor scavengers (red globules, hemoglobin) would prevent this effect. These agents indeed prevented growth inhibition, very likely because of the interaction of the furoxan ring with protein thiol groups. Therefore we used the non-thiol NO-scavenger PTIO, which, on the other hand, demonstrated to be uneffective, thus concluding that NO is not directly responsible of the antiproliferative effect.

We then tried different proteomic approaches aimed at assessing which is the portion of the furoxan able to inhibit SMC growth. For this purpose we selected as a model the 4-phenyl, 3-CN furoxan, one of the most active compound. Since we got positive feedback neither by 1D- and 2D-gel analysis coupled to mass spectrometry, nor by IodoTMT labelling (to evaluate S-nitrosylated

proteins), we recently utilized SILAC analysis to evaluate the possible different expression of proteins which regulate G1/S cell-cycle progression, after incubation with the furoxan. Of more than 700 selected proteins, we found a significant variation in the expression of 12 proteins. Among these, are nuclear factors involved in nucleic acids' replication and proteins regulated by SUMO1, whose reduced expression determines a block in G1 phase, due to cyclin-dependent kinases inhibition, thus possibly explaining furoxans' antiproliferative effect on SMC. We confirmed by western blot analysis that 4-phenyl, 3-CN furoxan also inhibited SUMO-1 expression, likely due to the nucleophilic attack at the 3-position by thiol groups present on the surface of this protein. On the other hand, PTIO, when co-administered with the furoxan, did not prevent the effect, thus confirming that NO-release is not involved in the antiproliferative effect. Even if we cannot exclude the participation of cellular proteins other than SUMO1, this evidence allows to consider furoxans (either alone or conjugated with other pharmacophores) as interesting antiatherosclerotic molecules.