Abstract

Cancer development is a complex pathological process that exploits a variety of biological actors. The identification of new molecular entities able to interfere with new biological targets, involved in tumorigenesis, is strongly needed, both for the development of new promising drug candidates, and, as chemical probes useful to further investigate less understood biological aspects. Two main targets, involved at different levels, in cancer development, have been thoroughly investigated: Macrodomain proteins, MacroD1 and MacroD2, and the Bcl-2 associated athanogene 3, BAG3 protein. The results obtained can be summarized in the two main sections, reported below according to the target of interest:

a) Discovery of new modulators of human Macrodomain proteins, MacroD1 and MacroD2, by structure-based and X-ray crystallography based approaches

MacroD1 and MacroD2 are two ortholog members of the epigenetic family of the Macrodomain containing proteins, which have been recently identified as attractive targets for the treatment of cancer, due to their well-established overexpression in several human tumors[1-2].These proteins can act as *erasers* of the histone code ADP-rybosilation, a post-translational modification involved in the modulation of gene expression and chromatin remodeling[3]. With the aim of identifying new modulators of these high related proteins, we performed two different drug discovery approaches, a structure based drug design on the MacroD2 crystal structure (PDB: 4IQY)[51] and a Fragment screening, X-ray crystallography based, on the protein MacroD1. Concerning the first approach, starting from the crystal structure of MacroD2 protein, in complex with ADP-ribose, its natural ligand, we performed a preliminary virtual screening on a Database of 16 million of 1,4 disubstituted triazoles. Results analysis allowed us to select the most promising molecules in terms of docking score and shape similarity. The next step consisted in the synthesis of the most promising molecules basing on a versatile and suitable synthetic strategy. The synthetized molecules were, then, tested in collaboration with the Structural Genomics Consortium of Oxford, to evaluate their ability to bind the target protein with Alpha Screen, Biolayer interferometry and Isothermal titration calorimetry. These biophysical methods allowed us to disclose compound **SP-2** as a real binder of the protein MacroD2, with a dissociation constant of 2.54±1.1 μM. This promising molecule will be further investigated on MCF-7 cancer cells, overexpressing the protein, to assess its potential antitumoral acitivity.

Concerning the study of MacroD1 protein, a fragment screening has been carried out, allowing the identification of three fragments co-crystallized with the protein MacroD1. Since with the surface

plasmon resonance (SPR) assay the binding to the protein was confirmed, for two of the three fragments, we decided to start to investigate, in silico, the binding mode of the most promising one, compound **3**, in order to develop a collection of high affinity binders of the target protein. These new fragments have been synthesized and then tested again by SPR, against the protein MacroD1 and in line with the computational predictions, four of them, showed to bind the target protein with higher affinity, compared to the lead compound; these results will open the way, to a deeper exploration of these fragments, as useful tools to develop new chemical probes able to modulate the protein MacroD1.

b) Design, synthesis and biological evaluation of the first BAG3-Hsp70, complex, inhibitor as an attractive candidate for the development of a new class of chemotherapeutics

BAG3 (Bcl-2-associated athanogene 3) is a multidomain protein, which, through its BAG domain, is able to interact with several partners, modulating, thus, key signaling pathways involved in physiological and pathological processes[4]. Indeed, BAG3 has been shown to sustain cell survival and resistance to chemotherapy, in human cancer cells, hence, this protein can be considered a therapeutic target of human malignancies[5]. With the aim of exploring this attractive target, basing on a *structure-based drug design* approach combined with biophysical methods, we screened a huge library of commercialy available molecules, against the BAG domain of the protein. Starting from the virtual screening and SPR results, we selected a 2,4 thiazolidindione endowed molecule (**BO-7**), as a promising BAG3 activity modulator. This compound, indeed, showed to bind with a good affinity to the full length protein (K_D: 5.2±3.8 nM) and to its isolated domain (K_D: 3.51±2.7 nM), with a good selectivity, since no binding affinity was measured for two other BAG proteins tested, BAG1 and BAG4. Then we decided to evaluate the potential antitumoral acitivity of the disclosed hit on A375 melanoma cancer cells, which are known to overexpress the BAG3 protein, and **BO-7** resulted to have a good cytotoxicity (25±1.5 μM) against the cell line tested, in line with our predictions. Starting from these promising outcomes, we developed a collection of synthetic 2,4-thiazolidindiones, as derivatives of the lead compound, that allowed us to identify a promising molecule (**LK-4**); this compound, infact, was able to selectively inhibit BAG3 protein, interfering with its BAG domain and interfering with the BAG3-Hsp70 protein-protein interaction. **LK-4**, indeed showed to bind with high affinity to the protein (K_D: 11.1±3.9 nM) and the isolated BAG3 domain (K_D: 6.4±2.2 nM) and, to have a high cytotoxicity (IC_{50}: 16±1.5 μM) against A375 melanoma cancer cells, and at the same time, a good selectivity; in particular, the disclosed inhibitor did not show to bind other BAG isoforms, BAG1 and BAG4, and, moreover, it did not affect the cell viability of PMBC human normal cells. Finally a co-immunoprecipitation assay confirmed that

LK-4 activity was mediated by the inhibition of the BAG3-Hsp70 complex formation, in cell, and in a time-dependent manner.