Abstract

Lysine acetylation of histone tails is an epigenetic hallmark that plays a crucial role in the regulation of chromatin structure. Alteration in acetylation levels and/or aberrant activity in proteins involved in this crucial regulation have been linked to the development of several human diseases. Bromodomains are protein modules that, through the recognition of the acetyl-lysine modifications, drive transcriptional programmes that result in phenotypic changes. In this *scenario,* BRD9 has been receiving growing interest in diverse therapeutic areas, particularly oncology. Indeed, acting as a subunit of the mammalian SWI/SNF (mSWI/SNF) chromatin remodeling complex, BRD9 regulates the expression of oncogenes and anti-apoptotic proteins leading to abnormal cell proliferation and survival in different tumor types. These findings stimulated an intensive research activity on BRD9 that emerged as an appealing target in cancer therapy. In addition, recently it has been assessed that targeting BRD9 could hold a potential for the treatment of inflammation.

On these bases, the present research work had been focused on the discovery and development of new agents able to disrupt the activity of BRD9 within the SWI/SNF complex by applying a multi-disciplinary strategy. Intense efforts and coordinated research work between the computational and the synthetic units led to the development of the first 3D structure-based pharmacophore models for BRD9 that represent valid and effective implements for the entire scientific community interested in the identification of new potent and selective ligands. Aided by these pioneering *in silico* tools, three new classes of compounds were effectively explored, supported by the application of modern synthetic methodologies that allowed rapid access to large collections of derivatives.

Abstract

At the beginning of this BRD9 project, an early and simplified version of the three-dimensional pharmacophores, namely the 4-point "pharm-fragment" model, was developed and employed for the design of a class of aryl sulfonamide-based compounds (**49-74**). Nevertheless, the poor activity obtained with these small molecules prompted a careful reevaluation of the computational tool. Therefore, the combination of iterative rounds of *in silico* design with effective syntheses of specific compounds and the final biophysical evaluations of the binding activity on BRD9, have been the cornerstone to optimize the efficiency of the compounds' selection process. Thus, two more precise models consisting of 7 pharmacophoric points were built. Specifically, the development of the "pharm-druglike2" model was crucial for the chemical exploration of a class of compounds featuring the 1-ethyl-[1,2,4]triazolo[4,3-*a*]quinoxaline scaffold. Deepened SAR studies, led to the identification of new interesting BRD9 ligands, specifically four compounds (**1**, **5**, **11**, and **42**) stood out for their potent binding on BRD9 in the low micromolar range and high selectivity across a panel of bromodomains. In collaboration with Prof. Irace (Università degli Studi di Napoli Federico II), compounds **1**, **11**, and **42** showed interesting bioactivity in interfering with tumor growth and proliferation, proving promising outcomes in leukemia cells.

Finally, the employment of the "pharm-druglike2" model, led to the discovery of the latest scaffold, *i.e.*, the 1-ethyl-1*H*-pyrazolo[3,4-*b*]pyridine core. The promising potential, owned by this new class of small molecules as new BRD9 ligands, was assessed by preliminary biophysical data of the hit compound **43**, which disrupted the recognition BRD9 – Histone H4 at 10 μM, presenting 55.0 ± 1.5 % residual BRD9 activity.

In parallel to the 3D pharmacophore-based discovery, two alternative approaches were explored to circumvent the crosstalk and complexes formation occurring between epigenetic modifiers. These mechanisms often represent a bottleneck for the achievement of a powerful and long-lasting anticancer activity of inhibitors that target specific subunits. Therefore, in addition to the conventional protein inhibition approach, we decided to explore the protein targeted degradation strategy. Herein it is reported a new class of Proteolysis Targeting Chimeras (PROTACs) taking advantage of our identified potent and selective ligand **1** for BRD9 engagement. Initial biological results carried out in collaboration with Prof. Altucci (Università degli Studi della Campania Luigi Vanvitelli), highlighted the promising degradation activity of the PROTAC compound **75**, which showed an evident depletion of BRD9 at 5 μM in U937 treated cells after 24 h, that was also reflected into a strong cytotoxicity in the same cell line.

Moreover, herein is reported the accurate design and the efficient synthesis of the first multi-target probe **81** that could be useful to start a yet unexplored investigation of the ncBAF SWI/SNF complex. The hybrid compound **81** was conceived with the aim to perturb, through a single agent, two crucial subunits belonging to the above-mentioned complex, and both involved in the onset of acute myeloid leukemia: BRD9 and SMARCA4.

As a conclusion of the intense investigations of the biological role exerted in cell environment by BRD9, thanks to a collaboration with Prof. Maione (Università degli Studi di Napoli Federico II), during this project it was assessed the striking anti-inflammatory activity featured by both BRD9 ligands (*e.g.*, compound **1**) and the degrader **75**. All the tested compounds proved a remarkable decrease of IL-6 and TNF-

α in J774 macrophage cells, paving a new interesting therapeutic applicability of BRD9 targeting agents.

Finally, in this Ph.D. project, deepened studies in the emerging protein degradation field were carried out at the University of Dundee (UK) under the supervision of Prof. Ciulli. The results obtained, through the syntheses of specific VHL ligand derivatives followed by both biophysical evaluation and the crystal structure solution of **MP-1-39** bound to VHL protein, could provide important steps forwards to understand the mechanisms of molecular recognition of different stereoisomers and open the rational design of new VHL-based PROTACs. In parallel, the optimization of the degradation profile and pharmacokinetic properties of Leucine-rich repeat kinase 2 (LRRK2) PROTACs for the therapeutic treatment of Parkinson's disease was carried out. Among a small set of newly synthesized degraders, compound **MP01088** qualified as an attractive starting point for future drug development, presenting a strong LRRK2 degradation in mouse embryonic fibroblasts already after 4 h of treatment.