Abstract

Target identification and mechanism of action studies of plant-derived compounds play a critical role in drug discovery. The knowledge of the bioactivity of natural compounds can lead to a number of advantages: first of all, it is possible to understand their full therapeutic potential; in addition, it can allow the further identification of their side-effects, their toxicity and also structure-activity relationships studies.

This research project is focused on a *Reverse Chemical Genetics* approach, which relies on the screening of libraries of plant small molecules (provided by the Department of Pharmacy (DIFARMA)-Bioactive Natural Products, University of Salerno (UNISA), Fisciano, Italy) able to bind specific target proteins and on the validation of the ligand/protein interaction. In my PhD project I focused on three protein targets, over-expressed in cancer and identified as potential markers in several tumor cell lines: Nucleolin, Heat Shock Protein 70 (Hsp70) and Heat Shock Protein 90 (Hsp90).

Nucleolin (NCL) is a multifunctional protein involved in many process such as DNA transcription, ribosome biogenesis and regulation of mRNAs of anti-apoptotic and antiproliferative proteins such as AKT1, Bcl2, p53. Firstly, a screening of *ent*-kaurane and *ent*-trachilobane library by *Cellular Thermal Shift Assay* (CETSA) on Jurkat (leukemia T cells) and HeLa (cervical carcinoma) was performed, obtaining as main ligand of Nucleolin the 6,19-dihydroxy-*ent*-trachiloban-17-oic acid (**12**) from *Psiadia punctulata* ((Vatke) Asteraceae). Full length Nucleolin/**12** interaction was validated in HeLa (cervical carcinoma) cells by CETSA and *Drug Affinity Responsive Target Stability* (DARTS). Nucleolin RNA Binding Domains 1-2/**12** interaction was investigated by *Saturation Transfer Difference* NMR (STD-NMR), WaterLOGSY and *Surface Plasmon Resonance* (SPR): no interaction was observed with these two domains of the protein. The mechanism of action of the selected diterpene was studied by Flow Cytometry (sub G_0/G_1 cell cycle arrest), WB analysis (reduction of intracellular AKT1 and Bcl2 levels and pNCL levels on the cell membrane), RTq-PCR (reduction of AKT1 and Bcl2 mRNAs), MTT (IC_{50}: 20 ± 1 μM), Protein Synthesis and Wound Healing assays in HeLa cells (reduction of 20% of migration). Therefore, the 6,19-dihydroxy-*ent*-trachiloban-17-oic acid (**12**) may be considered as a new promising modulator of Nucleolin.

The second target protein was the molecular chaperon Heat Shock Protein 70 (Hsp70). A diterpene library was screened by SPR assay, in order to select putative Hsp70 ligands. SPR results showed that the *ent*-7β-acetoxy,18-hydroxy-15α,16α-epoxikaurane (epoxysiderol or compound **27**) from *Sideritis* spp (Lamiaceae) interacts with Hsp70 (K_D: 54 ± 1.2 nM). Epoxysiderol ability to

modulate Hsp70 activity was assessed through MS (no covalent binding), DARTS and WB experiments. Moreover, epoxysiderol was tested on HeLa cells by MTT (IC_{50}: 20 ± 0.9 µM), Flow Cytometry (G_2/M and subG_0/G_1 cell cycle arrest), WB for its effect on the intracellular levels of Hsp70, Hsp90, and Hsp70 client proteins (reduction of pAKT1, p-p38 and p-JNK1) in HeLa cells, and by WB also for Hsp70 cytosolic and cell membrane levels (reduction of Hsp70 levels). Finally, ATPase assay (50% of reduction in dose-dependent manner) and molecular docking studies (interaction with the Hsp70 Nucleolide Binding Domain) were carried out. Therefore, in this study epoxysiderol was identified as a new Hsp70 inhibitor through cell-free and cell-based assays.

Another target object of study in this PhD project was the Heat Shock Protein 90 (Hsp90). Fusicoccane diterpenes from *Hypoestes forsskaolii* ((Vahl) Acanthaceae), abietane diterpenes form *Zhumeria majdae* ((Rech.f. & Wendelbo) Lamiaceae) and from different *Salvia* spp (Lamiaceae) were screened against Hsp90 by SPR and by MTT in HeLa, Jurkat and MCF7 cells, selecting the 18-hydroxyhypoestenone (**6**) and lanugon Q (**20**) as Hsp90 ligands. Subsequently, MTT assay was performed to investigate their cytotoxic and anti-proliferative activity: 18-hydroxyhypoestenone was the most cytotoxic in HeLa cells (IC_{50}: 18 ±1 µM), whereas lanugon Q showed higher activity towards MCF7 (IC_{50}: 20 ± 2 µM). In addition, Flow Cytometry and WB analyses were carried out: G_2/M cell cycle arrest and reduction of p-Cdc2, pAKT1 and pERK1 levels were observed in HeLa cells after treatment with **6** (10 µM and 20 µM for 48h); Decrease of pERK, pAKT, cyclin A was observed in MCF7 after 48h of treatment with **20** (18 µM). Selected diterpenes were also tested against Hsp90 by ATPase activity assay: dose-dependent reduction (40%) of hydrolysis was observed with compound **6** (1,5, 10 µM), while no inhibition was induced by **20**. Furthermore, molecular docking studies were implemented with compound **6**, and the computational analysis of the Hsp90/**6** interaction suggested a C-terminal domain. In conclusion, in this study 18-hydroxyhypoestenone and lanugon Q were identified as new Hsp90 interactors, able to modulate its activity and its client proteins levels.