Publishable summary

The purpose of this project was to train a highly qualified researcher (Dr. Reshma Rani) in managing a research line focused on the development of anti-cancer agents able to fight invasive tumours, which are normally resistant to chemotherapy and radiotherapy. The rationale of this project is based on the fact that highly invasive tumour phenotypes show a metabolic switch ("Warburg effect") from oxidative phosphorylation to an increased glycolysis. This change in glucose metabolism, in favour of a less efficient process for production of energy and anabolites, confers several advantages on the tumour cells, such as the ability to grow in poorly oxygenated conditions, which are typical of invasive hypoxic tumours. One of the key enzymes actively involved in anaerobic glycolysis, the muscle isoform of human lactate dehydrogenase (*h*LDH5), was shown to be overexpressed by metastatic cancer cells, and has been linked to the vitality of tumours in hypoxia. This enzyme is currently being considered as a valid target for new anticancer agents, since its inhibition leads to a cut in cancer energy supply, thus reducing its metastatic and invasive potential. A validation of *h*LDH5 as a safe target derives from the observation that in humans, hereditary *h*LDH5 deficiency causes a certain level of myopathy only after intense anaerobic exercise, whereas it does not provoke any symptoms under ordinary circumstances. This project will support a qualified international researcher in the management of a research line including molecular design and synthesis of a series of new compounds, as well as in the participation in the biological evaluation of their properties.

The researcher, was able to efficiently carry out the molecular design and synthesis of a series of new compounds, as well as to actively participate in the biological evaluation of their properties. Each step of his formation was adequately followed by additional specialized trainers: Dr. Tiziano Tuccinardi for the molecular modelling; Prof. Gino Giannaccini, for the enzyme inhibition bioassays; Dr. Valeria Di Bussolo, for the synthesis of gluco-conjugates.

The host institution had previously discovered a suitable structural scaffold, based on *N*-hydroxyheterocycles (NHIs), which has furnished some efficient *h*LDH5-inhibitors, possessing "first-in-class" potency. This scaffold constituted the basis for the development of larger series of optimized NHI-analogues, as well as for appropriate variations of the central NHI-scaffold, leading to completely new classes of *h*LDH5-inhibitors.

Initially, Dr. Rani, who had a very strong chemical background mostly focused on organic synthesis, started to learn more about medicinal chemistry, structure-activity relationships, pharmacophoric moieties, drug-likeness, and all other concepts that are crucial for the development of new therapeutics. She then participated to an extensive literature coverage of the most modern inhibitors of *h*LDH5, leading to the publication of a comprehensive review which was published in an internationally renowned peer-reviewed scientific journal [Granchi C, Paterni I, Rani R, Minutolo F, "Small-molecule inhibitors of human LDH5", *Future Medicinal Chemistry* **2013**, Vol. 5, Issue 16, Pages 1967-1991, DOI: 10.4155/fmc.13.151].

The researcher, then, efficiently worked on the optimization of some crucial steps involved in the synthesis of NHI-based *h*LDH5-inhibitors, including those that were conjugated to sugar portions, thus allowing their preparation on a relatively large scale. This was particularly important since gram-scale amounts of these inhibitors were needed for further pharmacological evaluation, which were made possible by the successful synthetic advances promoted by the researcher's work.

Some of these *h*LDH5-inhibitors proved to efficiently reduce proliferation and growth of highly invasive cancer cells, such as those deriving from pancreatic-ductal-adenocarcinoma (PDAC). This type of tumour is generally highly hypoxic and, therefore, resistant to radiotherapy and conventional chemotherapy, which is nowadays based on gemcitabine. We were pleased to find that, under hypoxic conditions (1% O_2), these *h*LDH5-inhibitors proved to be particularly effective in reducing cancer cell proliferation, with IC_{50} values as low as 0.9 μM. They also displayed synergistic interactions with gemcitabine, when used in combination in hypoxia. Most importantly, when tested on 3D tumour spheroids, a kind of model that more closely resembles the cellular arrangement in real tumours, these compounds were able to induce apoptosis and to reduce invasiveness and spheroid-growth (Figure 1).

All these results, which were obtained in a collaboration with the VU Medical Center (Amsterdam), led to a joint publication in a highly prestigious international journal [M Maftouh, A Avan, R Sciarrillo, C Granchi, L G Leon, R Rani, N Funel, K Smid, R Honeywell, U Boggi, F Minutolo, G J Peters, E Giovannetti, "Synergistic interaction of novel lactate dehydrogenase inhibitors with gemcitabine against pancreatic cancer cells in hypoxia", *British Journal of Cancer* **2014**, Vol. 110, Issue 1, Pages 172–182, DOI: 10.1038/bjc.2013.681].

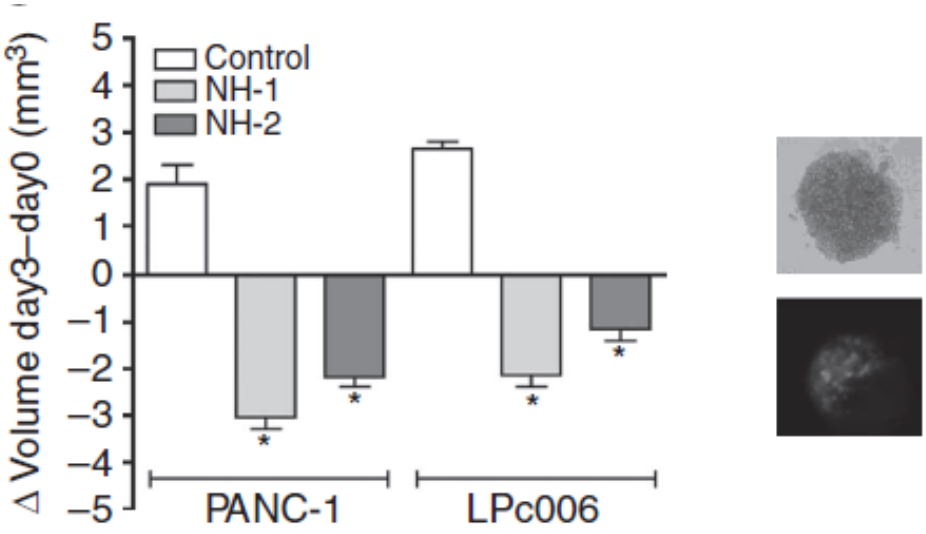


Figure 1. *Cytotoxicity of two NHI-based hLDH5 inhibitors in* ***3D-spheroids*** *deriving from pancreatic cancer cells.*

Another study was conducted in collaboration with the Universities of York and Bradford (UK), where these compounds were administered to human colon carcinoma HCT 116 cells. In these experiments these inhibitors caused an increase in the cancer cell ratio of NADH/NAD^+, since inhibition of *h*LDH5 (which uses NADH as the cofactor) leads to a reduced consumption of NADH. This effect caused a reduced activity of NAD^+-dependent deacetylase sirtuin 1 (SIRT1) and, therefore, to an increase in acetylated p53 (a widely established tumour-suppressor), which is known to be a target of SIRT1 deacetylation activity. In addition, activation of the redox-sensitive anticancer drug EO9 was enhanced in p53-positive cancer cells, attributable to increased activity of NAD(P)H-dependent oxidoreductase NQO1 (NAD(P)H quinone oxidoreductase 1). In fact, inhibition of *h*LDH5 (also indicated as LDH-A) increased EO9-induced DNA damage in these cancer cells, but importantly had no additive effect in non-cancer cells. In summary, this work indicates two distinct mechanisms by which suppressing LDH-A could potentially be used to kill cancer cells selectively, (i) through induction of apoptosis, irrespective of cancer cell p53 status and (ii) as a part of a combinatorial approach with redox-sensitive anticancer drugs via a novel p53/NAD(H)-dependent mechanism (Figure 2). These results were highly appreciated by the scientific community, as demonstrated by their publication in an newly emerging scientific journal (*Oncogenesis*) published by the prominent publisher *Nature Publishing Group – NPG* [SJ Allison, JRP Knight, C Granchi, R Rani, F Minutolo, J Milner, RM Phillips, "Identification of LDH-A as a

therapeutic target for cancer cell killing via (i) p53/NAD(H)-dependent and (ii) p53-independent pathways", *Oncogenesis* **2014**, Vol. 3, Article n. e102; DOI:10.1038/oncsis.2014.16].

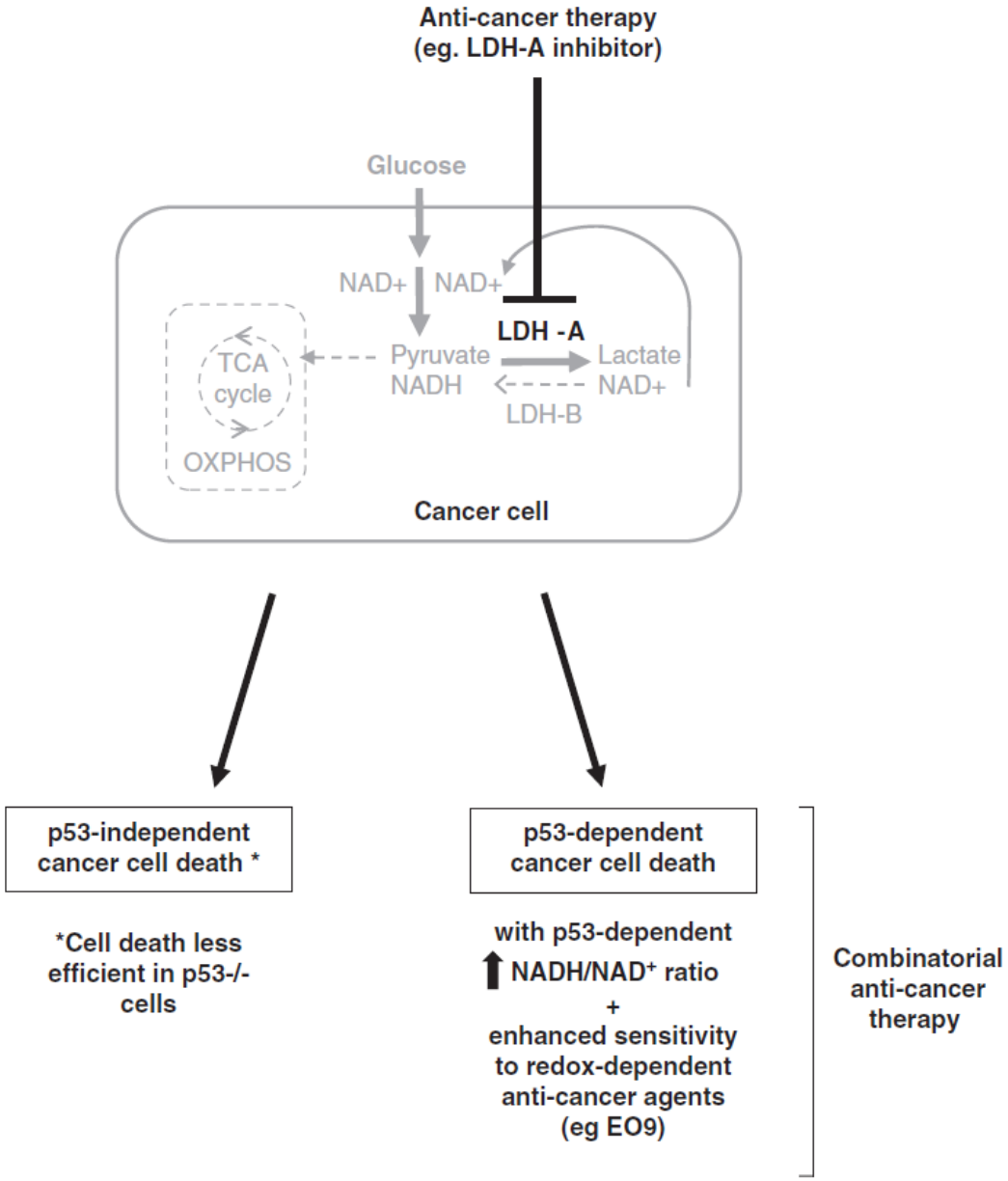
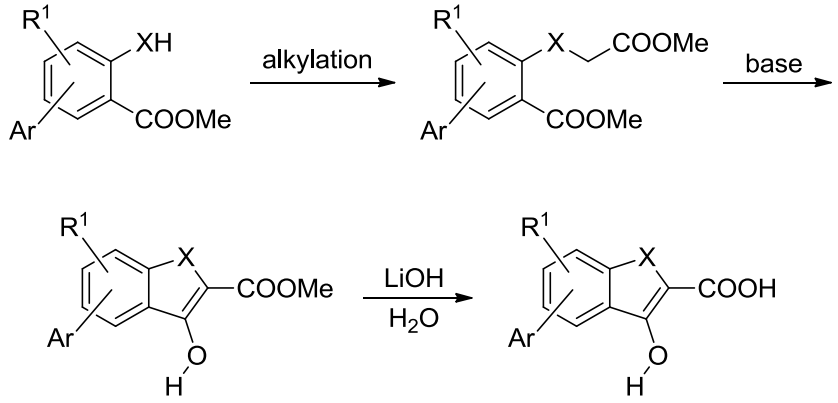


Figure 2. *Schematic representation of the effects caused by inhibition of LDH in perspective anticancer therapies.*

Subsequently, Dr. Rani produced large collections of analogues of NHIs, which possessed the same arrangements of the pharmacophoric OH and COOH/COOMe motif, but which differ from NHIs in their central scaffolds. In details, she worked on the implementation of efficient synthesis of 3-hydroxybenzofuran-2-carboxylates (BFR), 3-hydroxyindole-2-carboxylates (IND) and 3-hydroxybenzothiophen-2-carboxylates (BTP). The general synthetic sequence (Figure 3)

shows an initial alkylation of the nuclophilic XH group, followed by a base-promoted cyclization, which leads to the formation of the desired central scaffold. Final hydrolysis of the methyl ester produces the new perspective *h*LDH5-inhibitors.



	BFR	IND	BTP
X =	O	NH, N-Alk	S

Figure 3. *Synthesis of NHIs analogues BFR, IND and BTP.*

We were pleased to find a certain level of inhibition in all three classes of compounds, some of them displaying IC_{50} values in the low micromolar range. A docking analysis of some selected compounds into the X-ray structure of the LDH-A subunit of *h*LDH5 [PDB code: 1i10] showed that they occupy both the substrate binding pocket and a portion of the cofactor binding pocket.

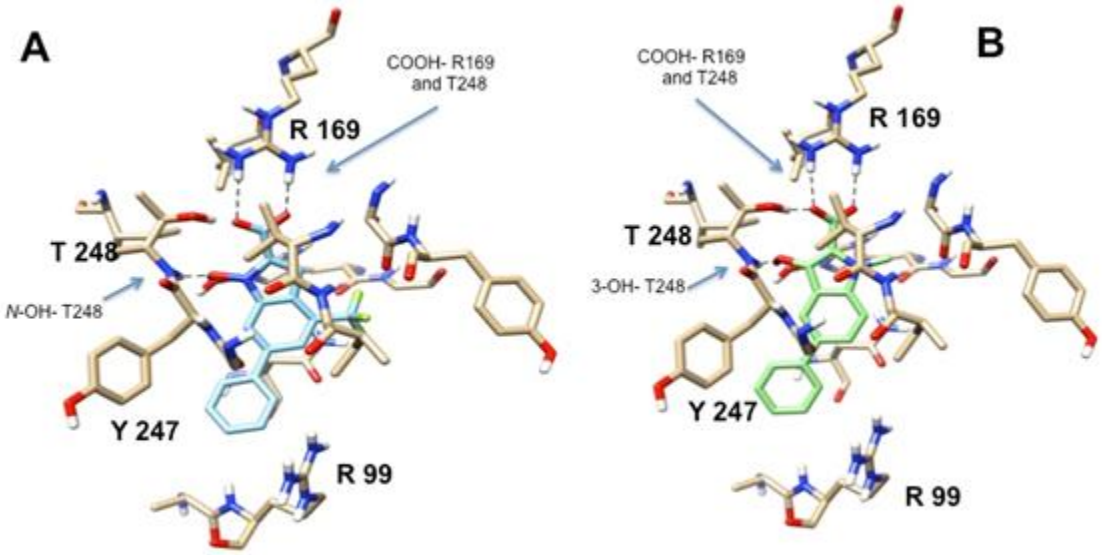


Figure 4. *Selected examples of NHI (A) and IND (B) inhibitors of hLDH5 docked in the enzyme active site.*

For example, a direct comparison of the docking of a previously developed NHI and a newly synthesized IND inhibitors of *h*LDH5 showed that their respective orientations in the enzyme active site are highly overlapped, and both compounds maintain similar interactions with some crucial aminoacid residues of the catalytically active site, such as those with Arg169 and Thr248.

Overall, this project effectively contributed to the production of *h*LDH5-inhibitors which display potent antiproliferative activities against invasive cancer cells. It is noteworthy that this antiproliferative effect is mainly caused by a selective cut of energy production in cancer cells. Therefore, thanks to their innovative and potentially safe mechanism of action, some of these compounds may constitute suitable pre-clinical candidates to be further developed as potential new and relatively non-toxic anti-cancer drugs.