Use of lentiviruses to infect human colorectal cancer cells with hERG1 channels –specific shRNAs: use in preclinical studies.

Background. Targeted therapies are changing the care and prognosis of many cancer patients. Many efforts have been made in this field through the identification of molecules and mechanisms that regulate tumour establishment and progression. Such molecules not only represent prognostic factors and predictors of benefits for chemotherapy, but have also been used as pharmacological targets for therapies. Recent evidence indicates that ion channels may represent novel targets for cancer therapy (reviewed in Arcangeli A. et al., Curr. Med. Chem, in press). Our and other groups have demonstrated that K^+ channels belonging to the hERG1 family can be included in the list of ion channels mis/overexpressed in cancer cells and whose activity is involved in the regulation of tumour establishment and progression. hERG1 proteins are encoded by the *herg1* (*human eag related gene1*) gene, that belongs to an evolutionarily conserved multigenic family of voltage-activated, outward rectifying K^+ channels, the *eag* (*ether a-gò-gò*) family. The *herg1* gene and the hERG1 protein, as well as the related current I_{hERG} , are expressed in neoplastic cell lines, as well as in several types of primary human cancers: endometrial adenocarcinomas, acute myeloid and lymphoblastic leukemias (AML and ALL), colorectal cancers, high grade astrocytomas; gastric cancers and precancerous gastric and esophageal lesions, such as the Barrett's oesophagus (Pillozzi S et al., Leukemia, 16: 1791-1798, 2002; Crociani O., et al., J. Biol. Chem, 278: 2947-2955, 2003; Lastraioli E et al., Cancer Res., 64: 606-11, 2004; Arcangeli A. 2005, *Wiley. Chichester (Novartis Foundation Symposium 266)* p 225-234; Masi A et al., *Br J Cancer* 2005, 93: 781-792; Lastraioli E et al., J Cell Physiol, 209: 398-404, 2006; Pillozzi S. et al., *Blood*, 2007,110:1238-1250).

hERG1 channels exert pleiotropic effects in the tumours where they are overexpressed: they control both cell proliferation and TNF-induced apoptosis. We also reported that hERG1 channels are important determinants for the acquisition of an invasive phenotype in solid cancers like colorectal cancers. In particular, colon cancer cell invasiveness *in vitro* strictly depends on the amount and activity of hERG1 channels. The regulatory role of hERG1 channels in cell invasion can be putatively traced back to a functional and physical association between hERG1 and β_1 integrins in neoplastic cells (Arcangeli A and Becchetti A. In: *Cell cycle in the central nervous system. Humana Press*. 2006, 81-94. Arcangeli A and Becchetti A., Trends Cell Biol., 16: 631-639, 2006).

Specific Aims. Based on the background explained above, we propose to deepen such studies in a project whose principal aim to use drugs and tools capable of inhibiting hERG1 channels as novel anticancer therapies in preclinical models. In particular, we propose to develop anti-hERG1 shRNA, inserted into lentiviral particles **(Lenti-anti hERG1-shRNAs)**. Such Lenti-anti hERG1-shRNAs will be used: a) to infect human colorectal cancer cells in order to stably silence hERG1 expression in cancer cells. Silenced cells will be further injected subcutaneously into nu/nu immunodeficient mice and the growth of colorectal cancer cells after hERG1 silencing will be evaluated; b) to treat mice harboring colorectal cancers (nu/nu mice injected subcuntaneously with human colorectal cancer cells), in order to evaluate the effect of hERG1 silencing as a novel antineoplastic therapy.

Experimental plan: 1) Identification and use of hERG1 specific shRNAs. Short hairpin (sh-) RNAs are double strand, 48 base-long oligomers consisting of two self-annealing sequences separated by a 6 nucleotide loop. When the loop is cleaved by cellular enzymes they become activated and trigger the endogenous RNA interference machinery, eventually leading to the degradation of the mRNAs whose sequence is identical to the shRNA. shRNAs are produced into mammalian cells by means of DNA vectors carrying a cDNA copy of the shRNA clone, downstream of the human U6 promoter, which is a target for RNA pol III. Commercial anti-HERG shRNA vectors (OPEN biosystems) will be first tested in HEK 293 over-expressing HERG channel. To do so, conventional delivery methods will be employed in this cell line, as high transfection

efficiency can easily be achieved with commercial cationic lipid preparations. Expression of HERG mRNA and protein in silenced cells will be compared to controls (non silenced, HERG-expressing HEK 293). The shRNAs with the highest silencing activity will be used in HERG-expressing colon cancer cell lines. **2) Production of colon cancer cell lines stably infected with Lenti-anti hERG1-shRNAs and *in vivo* experiments on nu/nu mice.** In order to obtain high levels of transfection in colon cancer cells, we will use lentiviral infection as a DNA delivery strategy. This strategy involves production of replication-imcompetent lentiviruses containing the desired shRNA vector. Before a stably transduced cell line expressing the shRNA of interest can be created, a lentiviral stock (containing the packaged transfer vector) will first need to be produced by co-transfecting the optimized packaging plasmid mix and the transfer vector construct into the TLA-HEK293T cell line. Co-transfection of the Trans-Lentiviral packaging mix and the transfer vector containing the shRNA of interest into TLA-HEK293T cells will produce a replication-incompetent lentivirus, which can then be transduced into the mammalian cell line of interest, colon cancer cells in our case. Once infected, these cells will not be able to assemble new viral particles, as they lack the required genetic information. Although such viruses are defective and cannot replicate in mammalian cells other than TLA-HEK293T packaging cells, a biosafety level 3 (BSL3) room is needed for the production of viral particles and infection of the target cell line. **To this purpose we will use the BSL3 room present at the Department of Public Health of the University of Florence.** Afterwards, biosafety level 1 is sufficient, therefore conventional cell culture hoods can be used. Stably infected colon cancer cell lines will be used for *in vitro* and *in vivo* experiments aimed at studying the effects of hERG channel ablation in these cells. Silenced cells will be compared to control cells in an array of *in vitro* assays in which cellular parameters such as proliferation, migration an adhesion to substrate will be measured. Afterwards silenced and control cells will be employed for *in vivo* experiments. In particular, silenced and non-silenced colon cancer cells will be injected subcutaneously into nu/nu mice ($1x10^3$ cells per mouse), house and handled under sterile conditions at the Laboratory of Genetic Engineering for the Production of Animal Models (LIGeMA) of the University of Florence. After five weeks mice will be sacrificed and tumor masses will be compared for size, presence of necrosis and other tumor-relevant features. **3) Intra-tumor injection of Lenti-anti hERG1-shRNAs.** Nu/nu mice will be injected subcutaneously with colorectal cancer cell lines as above. Seven days after injection (i.e. when subcutaneous masses become visible), mice will be divided in two groups: one group will be injected with **Lenti-anti hERG1-shRNAs** into the tumor mass, the other with control viruses, that is viruses carrying control plasmids. The two groups will be monitored for five further weeks and eventually sacrificed in order to compare key histological and molecular features, such as mass size, presence of necrosis, vasculature, etc as above. A BSL3 room is required during handling of viral particles and injection into mice. **To this purpose we will use the BSL3 room present at the Department of Public Health of the University of Florence.** Afterwards, because viral particles are replication-incompetent, mice are not infective and will be housed in the LIGeMA sterile rooms.

Expected results.

Our prediction, based on published results (see above and reviewed in Arcangeli A., et al., Curr. Med. Chem., in press) is that mice injected with hERG-silenced cells will show greatly reduced or retarded mass formation, with little angiogenesis or infiltration. When the efficacy of shRNAs will be proven in such preclinical model, we will go on to try and test the efficacy of Lenti-anti hERG1-shRNAs as antineoplastic therapy. We expect to obtain a reduction of tumor masses through the intratumoral injection of the Lenti-anti hERG1-shRNAs. If experimental results will fit well in our hypothesis, they will confirm the possibility of using hERG inhibiting tools as novel anticancer therapy.