CDP6 Recommendations concerning future work, if applicable (pp. 22-23)

Appendix to collective CDP6 response to SGA2 referees review report

30 July 2019

1. Link every output to the overall CDP objectives and HBP aims.

This work has the potential to transform current strategies for drug discovery in neurodegenerative diseases and is highly complementary to CDP6 aims without duplicating or overlapping work. Currently, it is difficult to target protein-membrane interfaces in part due to the absence of an established workflow and in part due to the limited literature that shows that this interface can be successfully targeted. **KRc6.4** has so far produced results and methodologies with substantial impact on:

- Understanding the physicochemical principles, with which small molecules are able to modulate protein-membrane interactions.
- Developed selective novel modulators of mutated PI3Kα, a target for glioblastoma patients.
- Developing methodologies for allosteric drug design for undruggable targets implicated in brain diseases

KRc6.4 is in close relation to SP6 by providing software tools, workflows, and molecular models, at the molecular level of detail to be included in the Brain Simulation Platform.

Additionally, in **KRc6.4** we are working together with SP8 and the Medical Innovation Platform data. Early achievements using the MIP include the identification of new subtypes of dementia subtypes and the first biological signatures for that disease, which allow a prognosis to be established before the onset of disease symptoms. We have received input for the epilepsy target from MIP and as the HBP website states (https://www.humanbrainproject.eu/en/medicine/), "We urgently need better diagnostic tools and treatments for brain-related diseases. People are living longer, thanks to improved sanitation, nutrition and treatments for infectious diseases.". Our **KRc6.4** outputs already are well aligned with the HBP scope describe above. Using input from the MIP platform we are in the process for devising new methodologies and bring new opportunities to drug design and development always in collaboration with neuroscience subprojects as well as with contributing atomic-level details molecular models to the ICT platforms as described above.

2. Show how the invention relates to novel compounds that are useful in medicine, possibly even in a personalized medicine pipeline and thus the potential to progress to the clinic and translate bench results to bedside.

The fact that a patent application stemming from CDP6 work has been deposited, proves that the goal of CDP6 towards developing target-based small molecule inhibitors for brain diseases has been achieved. This can be taken as a real proof of concept. Results of preclinical work demonstrating the relation of the invention to novel compounds that are useful in medicine, are demonstrated in section 3 below. These results are directly related to a personalized medicine pipeline as the discovered compounds have been designed and are proven to act selectively on the mutated H1047R PI3Ka protein in mouse models of cancer (see Figure 1 below and corresponding description). Thus, cancer patients are expected to be screened first for harboring the H1047R PI3Ka mutation and if the test is positive they would be eligible for receiving such a treatment, which follows the concept of personalized medicine.

Again, the work done on the glycine receptor, on the opiate, muscarinic and adenosine receptors and on the kinases involved the glioblastoma, among many others, are directly engaged in developing personalised medicine approaches. Yet at this stage we do not aim to deliver compounds of immediate used from patients but we wish to propose new targets and new prototypes of molecules which could then be used by pharmaceutical industry. The development of new categories of drugs demand time. The concept of allostery was proposed in 1961 (by one of us JPC) and today 50 years later there are 91 allosteric modulators available in the clinic.

A significant use-case for an integrated personalized medicine pipeline is the main aim of CDP6. All investigated CDP6 receptors such LGIC, GPCRs, kinases etc are directly engaged in human pathologies. Publications (by Zoe Cournia, Paolo Carloni, Giula Rossetti, Changeux & Cecchini etc) most often mention potential clinical applications (see CDP6 SGA2-12 months report).

3. Provide evidence for compound PI3K-010 that, with entering the next phase, a potential novel compound may be useful in medicine, including possibly in a personalized medicine pipeline

Note: The information below is confidential as it contains unpublished data

We have performed preclinical studies using mouse models of human cancer to evaluate the specific anti-cancer efficacy of compound PI3K-010 against mutated PI3Ka.

The inventors evaluated the *in vivo* activity of compound PI3K-010 and of PI3K-021, on mouse bearing heterotopic xenografts after subcutaneous injection of cells from the breast cancer MDA-MB-231 cell line, which is wild-type PIK3CA and of the breast cancer HCC1954 cell line, which bears exon 20 H1047R mutation on PIK3CA. For transplantation, eight-week old NOD/SCID mice were anesthetized using a steady flow of a mixture of isofluorane gas (4%) and oxygen and inoculated subcutaneously into their lateral flanks with 10^6 cancer cells and monitored daily until the tumors became palpable (tumor volume ~100 mm^3). The mice were then divided randomly into two groups for PI3K-010 or vehicle administration for a period of two weeks. Administration was achieved by mixing PI3K-010 or PI3K-021 with corn oil and subsequent oral dosing in 100mg/Kg doses twice a day. Average weights + S.E.M (n=7 for MDA-MB-231 and n-5 for HCC1954) are indicated. Figure 5

illustrates the gross appearance of surgically recovered PI3K-010-treated or PI3K-021 or untreated (control) xenografts.

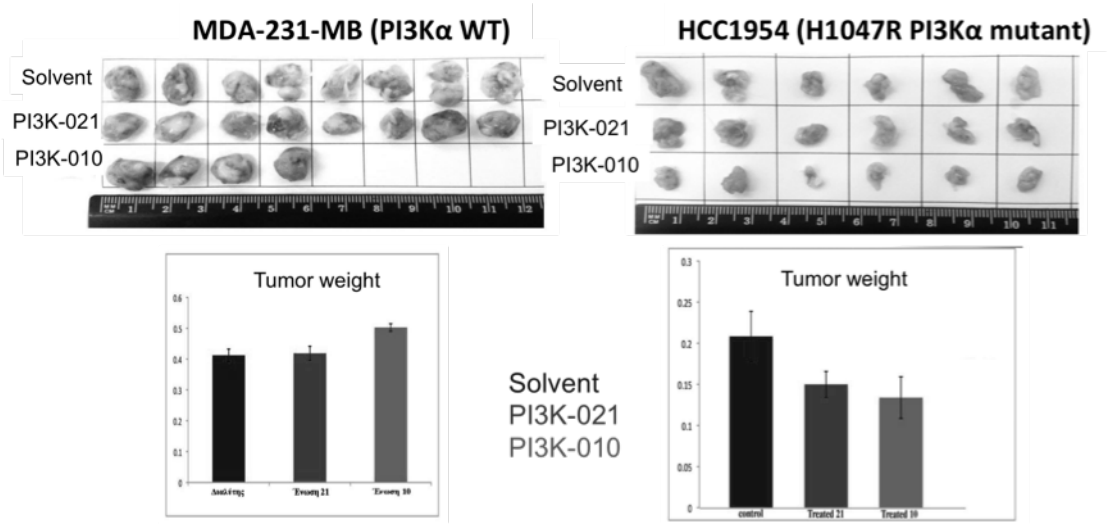

Figure 1. Results of PI3K-010 or PI3K-021 treatment of xenografts generated by heterotopic transplantation of the human breast cancer cell line MDA-MB-231 cell line, which is wild-type PIK3CA and of the breast cancer line HCC1954, which bears the PIK3CA mutation H1047R.

Additionally, PET/CT images of a WAPCre/exon20 Pik3ca*/MMTV-Myc mouse were acquired at the beginning and end of treatment (two weeks) of breast cancer with the PI3K-010 compound (cf. Figure 6). The compound was administered per os (by gavage) at 100mg/Kg doses. The control mouse received only solvent. The diminished uptake of 18F-FDG by remnants of breast cancer is evident. The white arrows indicate the anatomical sites of breast. High-level concentration of 18F-FDG in the urinary bladder (excretion of the tracer) and also in the heart is noted. To further study the effect of PI3K-010, we surgically removed breast tumors. Immunohistochemical analysis of the xenografts using an antibody against the cell proliferation marker Ki67 showed tumor necrosis and reduced proliferation after treatment in comparison with the controls (proliferation index 15.0+3.3% and 69.9+5.1%, respectively).

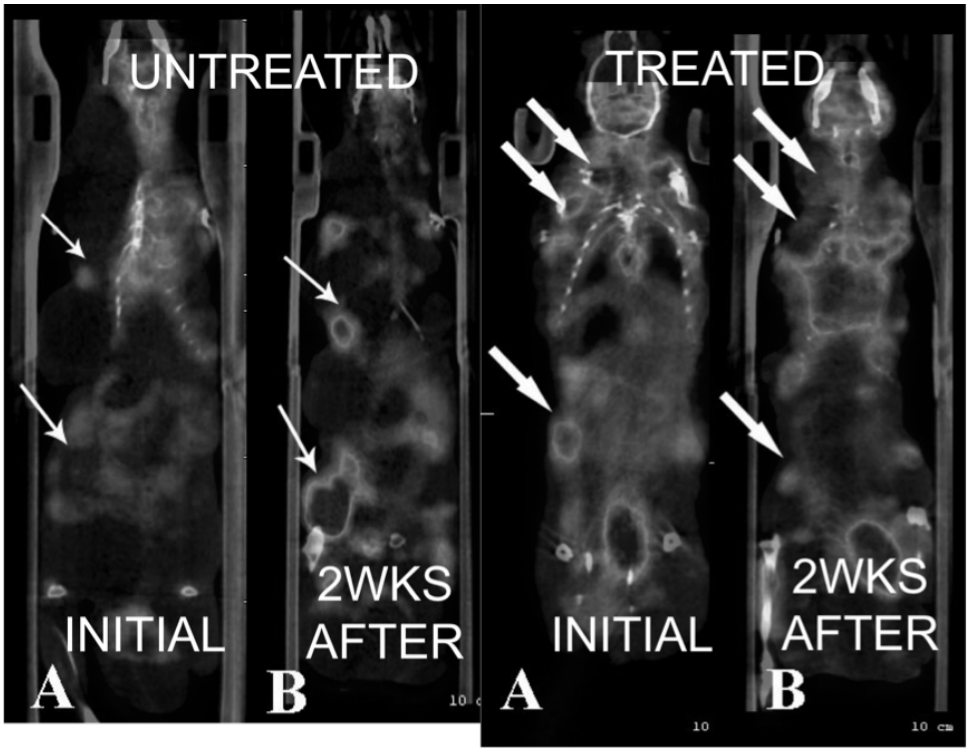

Figure 2. Example of micro PET/CT imaging of WAPCre/exon20 Pik3ca*/MMTV-Myc mouse (compared with a negative wild-type control) at the beginning and end of treatment (one month) of breast cancer with the PI3K-010 compound.

4. Consider further developing novel regulators of hotspot PI3Ka mutation E545K, including for neuronal plasticity.

We have indeed extended our simulations to assess the mechanism of the PI3Kα E545K activating mutation. Extensive Molecular Dynamics simulations were performed to examine conformational changes differing between the wild type (WT) and mutant proteins as they occur in microsecond simulations. In the E545K mutant PI3Kα, we observe a spontaneous detachment of the nSH2 PI3Kα domain (regulatory subunit, p85α) from the helical domain (catalytic subunit, p110α) causing significant loss of communication between the regulatory and catalytic subunits. We examine the allosteric network of the two proteins and show that a cluster of residues around the mutation is important for delivering communication signals between the catalytic and regulatory subunits. Our results demonstrate the dynamical and structural effects induced by the p110α E545K mutation in atomic level detail and indicate a possible mechanism for the loss of regulation that E545K confers on PI3Kα.

See Leontiadou *et al.*, "Insights into the mechanism of the PIK3CA E545K activating mutation using MD simulations." Sci Rep, 2018, 8(1):15544. https://www.nature.com/articles/s41598-018-27044-6

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From the simulations we extracted representative structures of both the WT and E545K mutated proteins and applied a small molecule binding cavity recognition algorithm. Binding cavities existing in the E545K mutant form but not in the WT protein were identified. A candidate binding cavity was thus selected in the mutant RIK3CA, which is located near the point of mutation. For that candidate binding cavity computer-aided drug design was performed through virtual evaluation of the database Maybridge (24,000 compounds) and ZINC (2,000,000 compounds) was performed. After post-processing the results for highest predicted free energy of binding score, physicochemical properties, toxicity, maximal chemical diversity, and pose viability, 20 compounds were selected from the Maybridge database and 22 were selected compounds from the ZINC database, which were purchased for in vitro evaluation.

PI3Kα activity assays were performed using PIP2 containing liposomes. Complexes of PIK3CA (WT or H10147R)/PIK3R1 were obtained from Millipore. The assay was based on a protocol provided by Millipore, with modifications. In this assay, PIP3 molecules produced by PI3Kα compete with biotinylated PIP3 for binding with recombinant GST-GRP1-PH domain (amino acids 263−380), which has been produced in bacteria, bound to glutathione coated 96-well plates. Quantitation of the competed amount of biotin-PIP3 is estimated by the peroxidase activity of streptavidin-HRP that binds to the plate and provides a measure for the activity of the protein. The inhibitors, PIK-108 or wortmannin, are preincubated with all the components of the assay mixture, except PIP2, for 10 min, at 25 °C. PIP2 is added in the end of the preincubation period, thereby initiating the kinase reaction. All assays were carried out in at least three independent experiments and each concentration in triplicate and at three concentrations: 25μM, 100μM, 200μM.

Based these assays three compounds (BRF-006, BRF-007 and BRF-008) were discovered to inhibit at least 50% of the mutated PI3K E545K (see Figure 3). Of particular interest is the fact that these compounds do not inhibit the wild-type kinase and therefore exhibit significant selectivity to the mutant E545K form. IC50 values will need now to be determined from dose−response curves using logit-log plots. If the leads are verified, the team is will continue with the optimization of these compounds for potency and selectivity against the mutant E545K form.

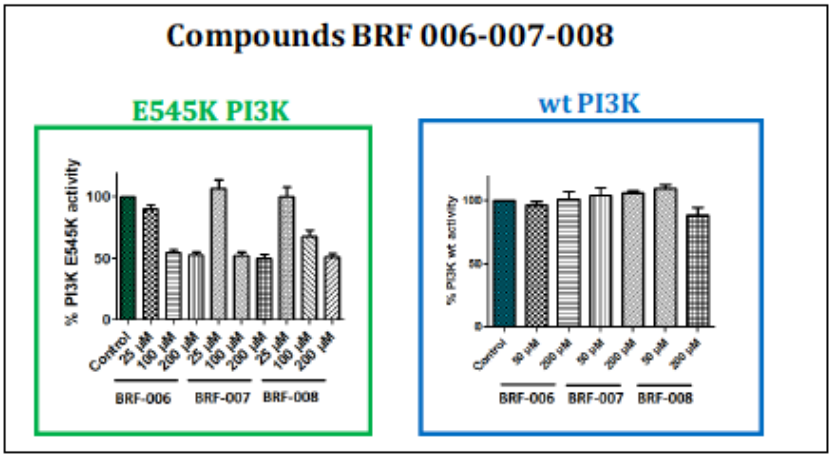

Figure 3. Cell-free in vitro assays of compounds BRF-006, BRF-007 and BRF-008 showing the inhibition of the mutated PI3Kα E545K and WT PI3Kα at three different concentrations.