PI: Barbara La Ferla

Project title: Development of anticancer druggable compounds from a chemically liable potent lead FR054.

Abstract

Cancer is one of the main cause of death worldwide. More precisely the second leading cause of death behind cardiovascular diseases, counting globally over 8.7 million deaths on 17.5 million cancer case in 2015 (Lancet. 2016;388(10053):1459-1544), and with an increasing trend. These numbers testimony that the "war on cancer" is far from being won, and that there is an urgent need to identify new therapeutic approaches. In this regard our multidisciplinary research team, in the past 4 years, has been exploring a potential therapeutic approach based on the inhibition of the Hexosamine Biosynthetic Pathway. We identified a potent promising lead compound, named FR054, able to inhibit a specific enzyme the PGM3/AGM1, present only in this pathway, thus reducing toxicity due to off target inhibition. This lead is able to inhibit the pathway in different cancer cell lines and showing encouraging efficacy in the treatment *in vivo* of MDAMB-231 xenografts mice with no noticeable toxicity. Nevertheless, the poor chemical stability of FR054, prevents the further development of this promising compound.

Within this project our main goal is to improve the chemical stability of the lead. This will be achieved by means of a combination of computational methods, for a structure-based virtual screening of a library of designed candidates, chemical organic methods for the synthesis of new chemical entities, and biochemical techniques for the biological activity evaluation.

Background, aims and significance of the proposed work

In the last 2-3 decades scientist have identified that many cancer cells present aberrant *N*- and *O*-linked protein glycosylation, and that these aberrant glycoconjugates play different roles in several steps of tumor progression regulating tumor proliferation, invasion, metastasis, and angiogenesis1,[1,2].

Based on these findings, the biosynthetic machinery at the basis of this post translational modification is now considered to be a hallmark of cancer, and thus is a promising target for the design and synthesis of antitumor drugs[4]. A key component of this machinery is the hexosamine biosynthetic pathway (HBP) responsible for the biosynthesis of uridinediphosphate *N*-acetylglucosamine (UDP-GlcNAc).

The possibility to target this pathway to specifically induce cancer cell death, also in combination with other metabolic inhibitors, may represent a novel and effective strategy.

In our preliminary work[3], a small library of potential inhibitors of AGM1/PGM3 was designed using an approach based on the substrate-product analogy. We identified a very promising lead, named FR054 was then tested *in vitro* on different cell lines with particular focus on the breast cancer cell line MDA-MB-231, and successively *in vivo* on MDA-MB-231 xenograft mice showing a significant capacity in reducing tumor growth[4,5].

The main drawback of this promising lead is it's chemical liability that does not allow it's development as drug candidate.

Specific aims of the project will be the following:

1) Design a small library FR054 analogues, and identify by virtual screening a set of potential inhibitors of the enzyme AGM1/PGM3.

2) Synthesize and characterize the selected compounds for their structure and chemical stability.

3) Assess the inhibitory activity of the compounds through enzymatic assay.

4) Evaluate the effects of the HBP inhibition in breast cancer cells MDA-MB-231.

Experimental plan

WP1: Optimization of the lead compound FR054 (compound **12**)

Design of a library of analogues of FR054 and screening based on the analysis of the docking poses within the substrate binding pocket of AGM1/PGM3, synthesis of the most promising ligands.

Task 1.1: Design of a library of FR054 analogues and in silico screening. Months **1-3**. (in collaboration with Prof. De Gioia)

Aim of this task is to design derivatives of the previously identified lead compound, oxazoline FR054. The chemical liability of the lead compound is principally due to the anomeric acetal that in aqueous conditions tends to hydrolyse, or that may undergo nucleophilic ring opening. To improve the chemical stability we will modify the structure by substituting the five member ring oxygen with a methylene group or a thiol atom (a Figure 8). We will also attempt to substitute the ring nitrogen with the more stable substituents, in order to avoid the presence of the imine functional group. This could be achieved by reduction to the corresponding amine or through the substitution of the nitrogen atom (b Figure 8). We will also consider other similar structure such as triazoline (c Figure 1), and the glycofused carbamates or urea derivatives (d Figure 1).

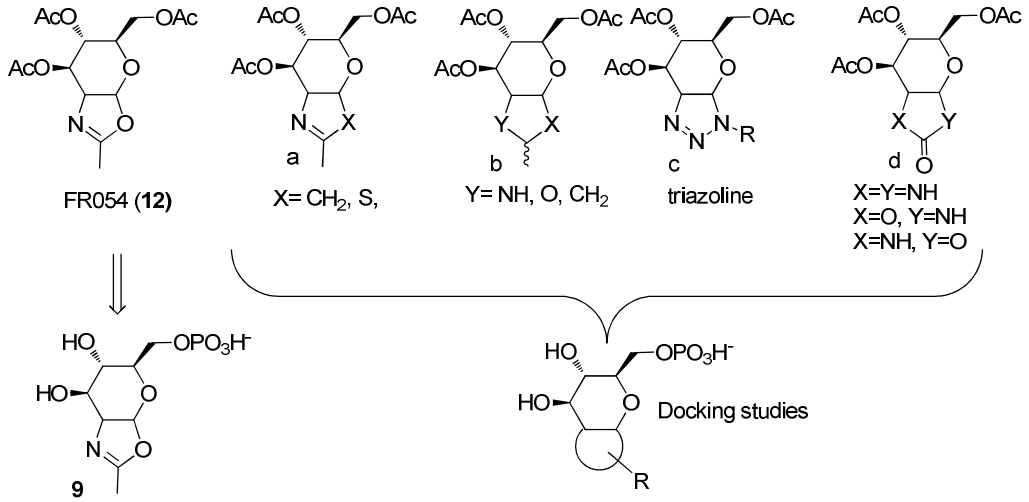

Figure 1: Library of analogues of the lead compound FR054, its active derivative **9**.

The in silico screening will be carried out on the deprotected and phosphorylated derivatives and will be compared to docking scores of FR054 active form compound **9**.

Molecular docking calculations will be carried out using the AGM1/PGM3 crystal structure from *Candida albicans* (Protein Data Bank code: 2dkc) cocrystallised with the natural substrate (GlcNAc-6-P). The docking scores will be computed with the Schrodinger 10.1 Maestro software, and the docking calculations will be carried out using the Glide docking module.[21] The force field applied will be OPLS 2005, which is preferred for biological systems and organic molecules.

The protein structure will be processed as previously done.[3] The docking of the potential inhibitors into the protein will be carried out assuming a protonation state compatible with pH 7, and sampling a box (18 Å × 18 Å × 18 Å) centred on the enzyme active site.

Task 1.2: Synthesis of best scoring members of the library: Months **2-30**.

The compounds identified in Task 1.1 as the best performing in terms of docking scores (in the number of 4/5) will be considered for the synthesis. Both the peracetylated compounds (analogues of lead FR054, Figure 8 a,b,c,d) and the deprotected derivatives will be synthesised, to be used in cellular assay (Task 2.2) and enzymatic assay (Task 2.1) respectively.

Methods for inhibitors preparation: Inhibitors will be synthesized according to usual organic chemistry procedures. Product purification will be performed by flash column chromatography on silica gel 230-400 mesh (Merck) or reverse phase if necessary. Molecules characterization will be performed by: ^{1}H and ^{13}C NMR spectra recorded with a Varian Mercury 400 MHz instrument; HR mass spectra will be recorded with a QSTAR elite LC/MS/MS system with a nanospray ion source. Purity of final compound will be checked by HPLC analysis obtained on a Jasco PU-2080 using Symmetry C18 HPLC column.

Task 1.3 Stability studies: Months **15-24**.

The stability of the synthesised compounds will be evaluated by NMR analysis of a solution of the test compound at different pH and in the presence of cellular medium. If necessary ^{13}C-labeled derivatives will be synthesized.

MILESTONES

1.1 Docking scores of the library of potential inhibitors. Month **3**.

1.2 Small library (4/5 compounds) of peracetylated and deprotected compounds. Month **18**. Expanded library: Month **36**

1.3 Stability analysis of the library members. Month **24**.

Pitfalls and caveats

We do not foresee particular risk in these two tasks; difficulties may arise during the synthesis of the compounds, therefore different synthetic strategies will be considered for each compound.

WP2: Biological activity screening of the synthesized compounds (done in collaboration with prof. Chiaradonna).

AIMS: In vitro biochemical evaluation of AGM1 enzymes activity in presence of the compounds. Toxicity evaluation and effect on HBP on cancer and normal cell lines.

Task 2.1: Enzymatic activity screening of the members of the library. Months **24-30**.

In order to test the in vitro inhibitory properties of the compounds generated from Task 1.2 we will used an enzymatic method already set up in our laboratory.[3]

Briefly, the method consists in evaluating the inhibitory ability of the selected compounds by measuring, using an HPLC-UV/Vis approach, the amount of UDP-GlcNAc produced after treatment with a cellular extract containing the complete enzymatic pool of the HBP pathway. UDP-GlcNAc represents the end product of the HBP pathway, and is obtained from GlcNAc-1P, the product of the PGM3/AGM1 catalysed reaction, and therefore the amount of this compound is correlated to the activity of AGM1. The screening will be carried out using a 1 mM concentration of the test compounds, and the amount of UDP-GlcNAc will be compared to a control reaction carried out without inhibitors.

Task 2.2: Cellular assays on selected active compounds: cell proliferation analysis and HBP evaluation: months **30-36**[4,5].

Upon identification of the best compound(s) (Task 2.1), compound's affinity for PGM3/AGM1 enzyme will be evaluated by using the cellular thermal shift assay (CETSA) and isothermal dose-response fingerprint (ITDRF) as previously described[4]. Briefly, both assays, by evaluating PGM3 stability in a dose- and temperature-dependent manner, will demonstrate the direct binding to the enzyme corroborating the enzymatic assay of Task 2.1. Since previous data demonstrated that FR054 has a strong effect on cell proliferation and survival of different cancer cells models such as MDA-MB-231 triple negative cancer cells, we will confirm the activity of the compound(s) by in vivo measuring its biological effects. In particular cell proliferation will be evaluated by MTT assay and trypan blue staining; cell death by Annexin V/Propidium iodide double staining. For these set of experiments we will use MDA-MB-231 cells and hTERT immortalized human mammary epithelial cell line (HME-1) as control of normal cells. In this set of experiments compound FR054 will be used as control.

An important characteristic of FR054 is the ability to induce UPR activation. Given that, new compound(s) will be tested also for their ability to induce UPR by two different approaches. First, by western blot analysis of UPR activation by measuring the level of phosphorylation or expression of proteins such as Bip, elF2a, ATF4 and in particular the pro-apoptotic protein Chop. Second, UPR activation will be in vivo monitored by using an MDA-MB-231 cell model engineered to express a CHOP promoter (-647 to +136) fused to fluorescent protein Cherry. Upon clone selection and in vitro and in vivo validation, these cell model will be useful to test the different compounds and to perform a rapid screening to identify the appropriate time and dose for each compound.

MILESTONES

2.1 Inhibitory activity of the tested compounds towards the production of UDP-GlcNAc. Month **30**.

2.2 Validation of lead modulators of HBP by enzymatic assays, cell growth analysis and HBP evaluation. Months **36.**

Pitfalls and caveats

We do not foresee particular pitfalls for this part of the project.

Expected results:

The main final goal consists in the identification of new compounds, analogues of the lead FR054, with an improved chemical stability, to be developed as a new anticancer drugs.

The obtained compound, inhibitors of the HBP pathway, will also represent new molecular tools to be used by researchers working in the field.

Feasibility and financial support

The organic chemistry research laboratory is fully equipped for the synthesis and characterization of the designed compounds, for the stability studies.

The biological evaluation will be carried out in collaboration with prof. Chiaradonna, with whom we have an established evaluation.

The research group has financed projects 2017-ATESP-0013, 2017-ATE-0257, 2018-ATE-0051, 2019-ATE.

Literature references

1. Dennis, J. W., Nabi, I. R. & Demetriou, M. Metabolism, cell surface organization, and disease. Cell 139, 1229–1241 (2009).
2. Lynch, T. P. & Reginato, M. J. O-GlcNAc transferase: a sweet new cancer target. Cell Cycle 10, 1712–1713 (2011). Pinho, S. S. & Reis, C. A. Glycosylation in cancer: mechanisms and clinical implications. Nat. Rev. Cancer 15, 540–555 (2015).
3. A. Paiotta, G. D'Orazio, R. Palorini, F. Ricciardiello, L. Zoia, G. Votta, L. De Gioia, F. Chiaradonna, B. La Ferla. Design, Synthesis and preliminary biological evaluation of Glc*N*Ac-6P Analogues for the Modulation of Phosphoacetylglucosamine Mutase 1 (AGM1/PGM3). Eur. J. Org. Chem, 1946-1952 (2018).
4. F. Ricciardiello, G. Votta, R. Palorini, I. Raccagni, L. Brunelli, A. Paiotta, F. Tinelli, G. D'Orazio, S. Valtorta, L. De Gioia, R. Pastorelli, R. M. Moresco, B. La Ferla, F. Chiaradonna. Inhibition of the Hexosamine Biosynthetic Pathway by targeting PGM3 causes breast cancer growth arrest and apoptosis. Cell death and disease, 9(3), 377 (2018).
5. F. Chiaradonna, F. Ricciardiello, G. Yang, R. Palorini, Q. Li, M. Giampà, F. Zhao, L. You, B. La Ferla, H. De Vitto, W. Guan, J. Gu, T. Zhang, Y. Zhao. Hexosamine pathway inhibition overcomes pancreatic cancer resistance to gemcitabine through Unfolded Protein Response and EGFR-Akt pathway modulation. *Oncogene* (2020), in press

PI recent papers

1) A. Paiotta, G. D'Orazio, R. Palorini, F. Ricciardiello, L. Zoia, G. Votta, L. De Gioia, F. Chiaradonna, **B. La Ferla.*** Design, Synthesis and preliminary biological evaluation of GlcNAc-6P Analogues for the Modulation of Phosphoacetylglucosamine Mutase 1 (AGM1/PGM3). *Eur. J. Org. Chem* 2018, 1946-1952.
2) F. Ricciardiello, G. Votta, R. Palorini, I. Raccagni, L. Brunelli, A. Paiotta, F. Tinelli, G. D'Orazio, S. Valtorta, L. De Gioia, R. Pastorelli, R. M. Moresco, B. La Ferla, F. Chiaradonna. Inhibition of the Hexosamine Biosynthetic Pathway by targeting PGM3 causes breast cancer growth arrest and apoptosis. *Cell death and disease*, 9(3), 377 (**2018**).
3) G. D'Orazio, A. M. Martorana, G. Filippi, A. Polissi, L. De Gioia, **B. La Ferla*.** N-Spirofused Bicyclic Derivatives of 1-Deoxynojirimycin: Synthesis and Preliminary Biological Evaluation. *Chemistry Select* (2016), 1, 2444-2447.
4) C. Airoldi, G. D'Orazio, B. Richichi, C. Guzzi, V. Baldoneschi, L. Colombo, M. Salmona, C. Nativi, F. Nicotra, **B. La Ferl**a*. Structural modifications of cis-glycofused benzopyran compounds and their influence on the binding to amyloid-β peptide. *Chemistry-An Asian Journal*. (**2016)** 11(2), 299-309.
5) G. D'Orazio, L. Colombo, M. Salmona, **B. La Ferla***. Synthesis and Preliminary Biological Evaluation of Fluorescent Glycofused Tricycle Derivatives of Amyloid β Peptides Ligands. *Eur. J. Org. Chem* (**2016),** 1660-1664.