Deliverable 4.1-2:

Group of hit compounds no.2&3

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Deliverable	**Number**	4.1-2	**Title**	*Group of hit compounds no. 2&3*
GT	**Number**	4	**Title**	Identification of active compounds for the treatment of obesity and diabetes with licensing potential
Action	**Number**	4.1	**Title**	Identification of hit compounds no.3

TABLE OF CONTENTS

LIST OF TABLES & FIGURES

1. Introduction

Metabolic disorders are the cause of several human diseases, such as obesity or type II diabetes that despite several recent advances still lack of effective therapies. The proposed strategy is based on the research results obtained by two of DIOmed project partners, during last years (IRB and INSERM). In this way, 3 identified proteins (Mitofusin-2, semicarbazide sensitive amine oxidase (SSAO) and diabetes and obesity related gene protein (DOR)) which can play a major role in obesity and/or diabetes will be taken as a basis.

This deliverable reports the results obtained in the screening for SSAO inhibitors and DOR expression modulators.

Hydrogen peroxide (H_2O_2) is known as an insulin–like agent, able to activate glucose transport in adipocytes. However H_2O_2 belongs to the family of reactive oxygen species suspected to participate in the insulin-resistance in type 2 diabetes. In white adipose tissue (WAT) the enzyme "semicarbazide-sensitve amine oxidase/vascular adhesion protein -1" (SSAO/VAP-1) is able to produce H_2O_2 once activated by addition of exogenous substrates, resulting in increased glucose uptake (Zorzano et al., Biochimica et biophysica acta 2003). On the other hand, the genetic invalidation of the AOC3 gene coding for the SSAO/VAP-1 results in a diminished infiltration of immune cells in fat depots (Bour et al., Am J Pathol, 2009), therefore limiting the "low grade inflammation "of adipose depots, which is suspected to favour the onset of insulin resistance (Lumeng et al., J Clin Invest 2007).Such duality between SSAO/VAP-1 activity and the adipose tissue physiology has an interest for obesity and diabetes treatments whether novel and selective SSAO/VAP-1 substrates or inhibitors can be developed.

DOR gene expression is high in skeletal muscle, heart and brain and specifically in muscle is highly repressed in Zucker Diabetic rats (ZDF) compared to lean non diabetic animals (Baumgartner et al., PLoS ONE 2007). In addition, unpublished data indicate that DOR expression is repressed in skeletal muscle and in adipose tissue from type 2 diabetic patients. Muscle DOR gene is also repressed in obese subjects. These data suggest that DOR may participate in the physiopathology of metabolic disorders such as type-2 diabetes or obesity. Furthermore, a single nucleotide polymorphism (SNP) named DOR1 and located in the human DOR promoter region associates with insulin resistance and with type 2 diabetes.

To find new active hits compounds for new targets, HTS of large chemical libraries of compounds is a proven way to identify novel chemical entities that target a biological system of interest. Generally, high throughput screening involves modern robotics, sophisticated control software, advanced liquid handling, and

sensitive detection methods. Through this process one can rapidly identify active compounds, which can provide a starting point for a drug design. This is the approach used in this project through the HTS unit of the BioFarma partner at the University of Santiago de Compostela (USC).

To this purpose, USC miniaturize the methodologies available at research groups in INSERM and IRB Barcelona of the new targets that are proposed to be involved in obesity and type II diabetes for HTS (USC). In order to study in front of them, the Prestwick Chemical Library, this contains 1.120 small molecules with a high degree of drug-likeliness screened against the three proteins.

The strategy consists of submitting to the target only a limited number of highly diverse drug molecules for which bioavailability and toxicity studies have already been performed and which have proven usefulness in humans. This initial screening will provide hits that will then be used as starting points for a drug optimization program which will rely on medicinal chemistry expertise.

2. Objectives

The general objective was to obtain hits that can be used to develop SSAO inhibitors and DOR expression modulators with potential use in the treatment of the Metabolic Syndrome.

The specific objectives of this GT were:

- To perform the High Throughput Screening (HTS) for the identification of compounds with capacity to inhibit SSAO activity and the further confirmation of positive hits.
- To perform the High Throughput Screening (HTS) for the identification of compounds with capacity to modulate the DOR expression, and the further confirmation of positive hits.

3. Results

Screening and identification of compounds with capacity to inhibit SSAO activity.

The screening was done with the advice of research group in IRB Barcelona, coordinated by Dr. Antonio Zorzano, at the University of Santiago de Compostela since they have operative HTS facilities. The Prestwick Chemical Library® contains 1.120 small molecules, 90% being marketed drugs and 10% bioactive alkaloids or related substances, thus it presents a high degree of drug-likeliness. The compounds that compose the library have been selected for their high chemical and pharmacological diversity as well as for their known bioavailability and low toxicity in humans.

The high number of diverse molecules in the Prestwick Chemical Library necessitates the use of robust assays with good sensitivity and a good dynamic range to maximize reliability and reproducibility for its use in our HTS program.

The assay for the regulation of SSAO activity was optimized for running in a miniaturized, 96-well plate format.

Adipocytes from human donors were used to screen possible inhibitors of SSAO. The HTS was carried out by measuring their ability to modulate H_2O_2 production by human adipose tissue homogenates. The H_2O_2 formation was quantiated by amplex Red (AR), which is optically non active regarding fluorescence, but when oxidized, e.g. by peroxidase, it is transformed in resorufin which emits signal. The quantity of H2O2 present in an unknown milieu is proportional to the Resorufin generated by AR oxidation.

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To this end, adipose tissue was homogenated and was plated in a 96-well plate (70 µg protein/well) and incubated for 30 minutes at 37ºC in the presence of the compounds of the Prestwick Chemical Library® at a concentration 10 µM and of the cromogenic mixture (Amplex Red and peroxidase). Each molecule was studied at a single dose of 10 µM and positive hit compounds were defined as those ones that induce an inhibition in the SSAO activity higher than 50%, compared to vehicle treated samples. All plates contained vehicle treated samples and benzylamine-treated samples as positive and negative controls of inhibition of SSAO activity, respectively.

Regulation of SSAO activity induced by tested compounds ranged from compounds that did not inhibit SSAO activity to compounds that inhibited SSAO activity in a 98%. Compounds showing activity higher than 50% of inhibition were considered as hits and were confirmed in an independent assay.

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The hits obtained are summarized in Table 1.

Compound code	DCI	% activity (10 µM) assay 1	% activity (10 µM) assay 2	Mean±eem
Prestw-170	Phenelzine sulfate	98.98	97.06	98.02±1.36
Prestw-553	Pentamidine isethionate	63.00	40.82	51.91±15.68
Prestw-868	Acetaminophen	55.22	33.18	44.20±15.59
Prestw-940	Esculetin	55.54	33.80	44.67±15.37
Prestw-1069	Mesalamine	56.19	40.55	48.37±11.05

Table 1: Hits identified as SSAO inhibitors in the HTS campaign and confirmed in a secondary screening.

The quality of the HTS campaign was controlled by measuring the signal/noise ratio distribution and Z' scores across the plates (Figures 1 and 2).

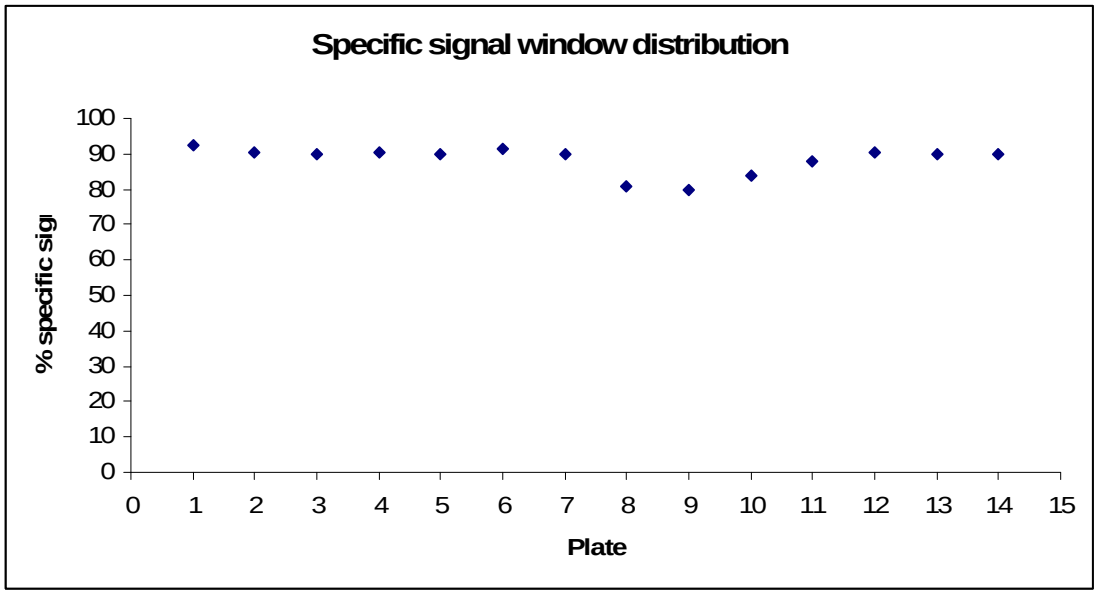

Figure 1: Signal/noise ratio distribution (expressed as % of specific signal window) across the plates studied.

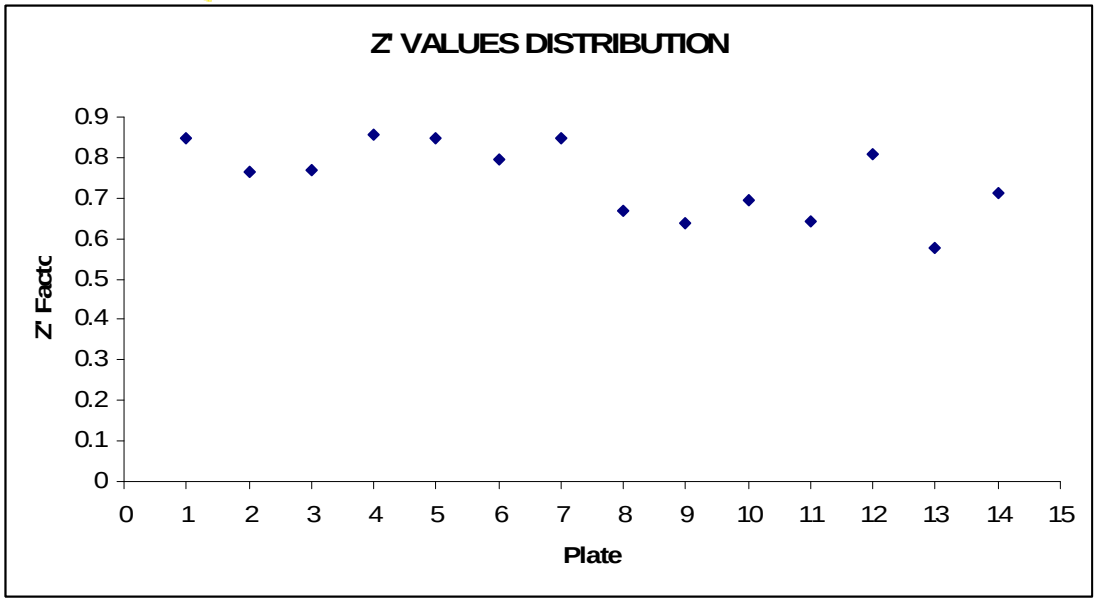

Figure 2: Z' score distribution across the plates studied. Z' scores is a measurement of data spread and its values for a robust assay should be always higher than 0.5. (Zhang J, Chung TDY, Oldenburg KR. A simple statistical parameter for in evaluation and validation of High Throughput Screening assays. Journal of Biomolecular Screening 1999; 4:67-73)

Screening and identification of compounds with capacity to modulate DOR expression.

The screening is done with the advice of research group in IRB Barcelona, coordinated by Dr. Antonio Zorzano, in collaboration with Dr. Mabel Loza at the University of Santiago de Compostela since they have operative HTS facilities. The Prestwick Chemical Library® contains 1.120 small molecules, 90% being marketed drugs and 10% bioactive alkaloids or related substances, thus it presents a high degree of drug-likeliness. The compounds that compose the library have been selected for their high chemical and pharmacological diversity as well as for their known bioavailability and low toxicity in humans.

The high number of diverse molecules in the Prestwick Chemical Library necessitates the use of robust assays with good sensitivity and a good dynamic range to maximize reliability and reproducibility for its use in our HTS program.

The assay for the regulation of DOR expression was optimized for running in a miniaturized, 96-well plate format.

The HeLa-pGL4 clone 57 was used to screen possible activators of the human DOR promoter. The HTS was carried out performing the luminescence measures of the luciferase activity 4 minutes after the addition of the luciferine using an integration time of 100 ms in a Tecan Ultra Evolution detector.

To this end, cells were plated in a 96-well plate (40.000 cells/well) and incubated for 16 hours in the presence of the compounds of the Prestwick Chemical Library® at a concentration 10 µM. Cells were incubated in parallel with T3 at 100 nM, which was used as a positive control and with DMSO (1%), which was used as a negative control.

The screening has been partially done (560 compounds) and we obtained a low signal/basal window, which resulted in low Z' scores values and therefore invalidated the data obtained up to now. We are currently refining the automated methodology in order to obtain better quality control parameters that allow us to correctly ascertain the activity of the compounds over DOR expression.

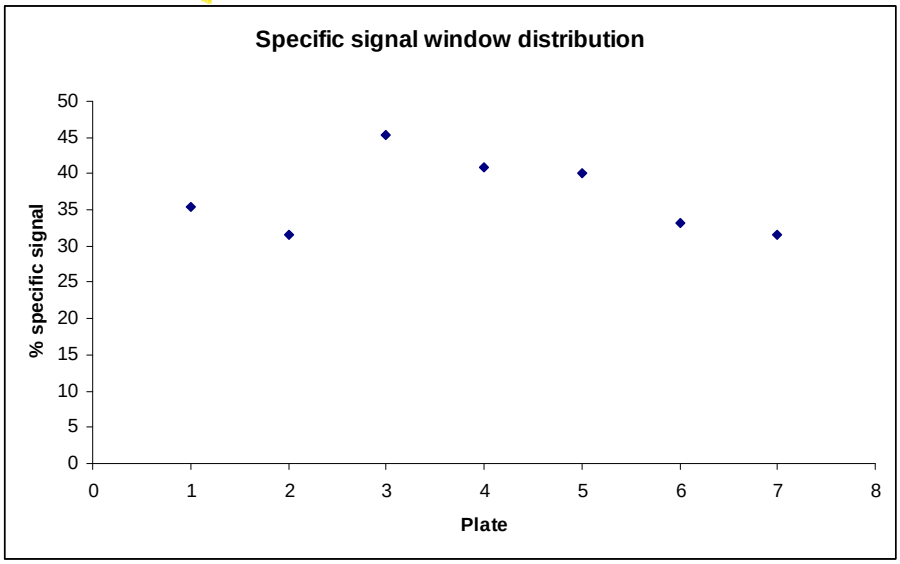

Figure 3: Signal/noise ratio distribution (expressed as % of specific signal window) across the plates studied.

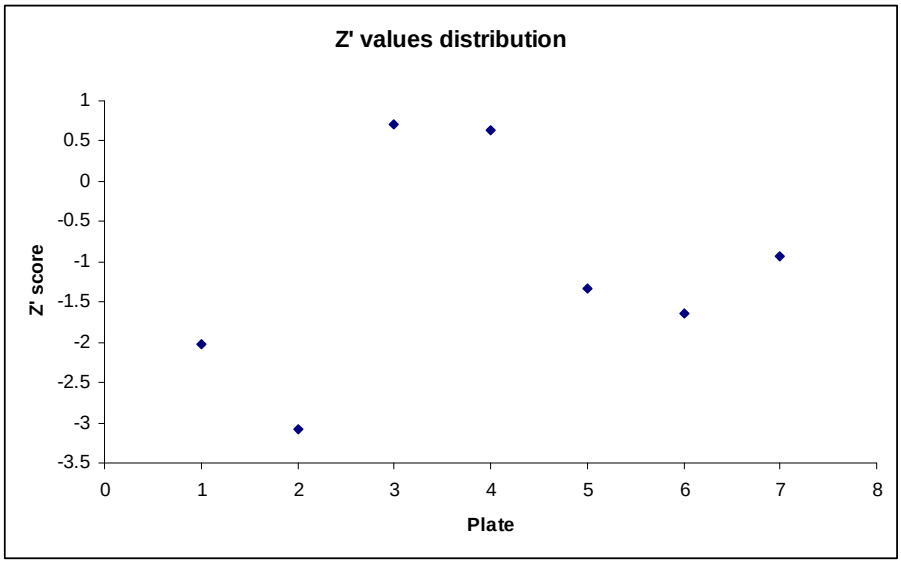

Figure 4: Z' score distribution across the plates studied. Z' scores is a measurement of data spread and its values for a robust assay should be always higher than 0.5. (Zhang J, Chung TDY, Oldenburg KR. A simple statistical parameter for in evaluation and validation of High Throughput Screening assays. Journal of Biomolecular Screening 1999; 4:67-73).

Conclusions

- 1120 compounds from Prestwick Chemical Library were screened with standard quality controls over SSAO activity;
- 5 compounds were identified as possible inhibitors of the human SSAO activity. The activity of these compounds is going to be further characterized at INSERMN;
- 560 compounds from Prestwick Chemical Library were screened with standard quality controls over DOR expression;
- The results obtained were rejected due to bad quality control parameters. Currently, the methodology is being fine-tuning in order to improve this assay and screen the Prestwick chemical library.