Indo Swiss Joint Research Programme (ISJRP)
RESEARCH FELLOWSHIP

EXCHANGE GRANT REPORT

Grant No.: RF23

PART 1 - GENERAL INFORMATION

Project Title: Functional characterization of cis acting elements in the Promoter region of CYP2C19

Keywords: CYP2C19, Promoter, transcription factor, transcription

Research area: Gene regulation- Basic Research

Start date: August 29, 2010.

Duration: 2 months

PART 2 - EXCHANGE PARTICIPANT(S) DETAILS

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2.3 Hosting scientist

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PART 3 - SCIENTIFIC & TECHNICAL INFORMATION

3.1 Purpose of visit:

1. To exchange expertise for exploring the transcription factors and their binding sites interaction in a promoter region of a gene using gel shift assays or electrophoretic mobility shift assays.
2. To undergo training in *in silico* analysis of the enzyme and substrates interaction to predict the inhibitory capacity of a substrate.

3.2 Short description of work carried out during the visit:

The work plan was modified slightly to optimize the methodology described in our proposal for effective and timely completion of the work, but the objectives remained the same. We used Cy-3 labelled oligos (labelled on 5’-end of forward strand of each double strand oligo) for binding sites instead of the biotin/P\textsuperscript{32} labelled oligos, and thus we avoided usage of hazardous radioactive material i.e. P\textsuperscript{32}and avoided the blotting step with biotin labelled oligos. Trial experiments with HepG2 and HeLa nuclear extracts have shown that the usage of Cy-3 labelled oligos is sensitive enough to detect the DNA-protein interactions in a gel shift. Briefly, the following work has been being carried out.

1. Purchase and annealing of the single stranded oligos to form a double stranded oligos representing the binding site of transcription factor. The list of additional oligos used for creating binding site for transcription factors other than the ones described in the proposal are given in Table 1.
2. HepG2 cell lines were maintained till 70-80% confluent and nuclear extract was prepared as the protocol mentioned in the proposal and was quantified for amount of proteins using Bradford reagent.
3. Standardisation of the gel shift assays has been carried out with varying amount of protein, poly dI.dC, and salt concentration as mentioned in the proposal.
4. Gel shift assays for the predicted transcription factors from CYP2C19 promoter region as mentioned in Table 1 has been carried out, with super shift assays for CCAAT enhancing binding protein beta (CEBPB) and Cyclic AMP responsive element binding protein (CREB) binding sites.
5. *In silico* analysis of CYP2C19 and its substrates has been carried out using the Swissdock software developed at the Swiss institute of bioinformatics, and the results were cross-validated with the autodock and autodockvina softwares.

Table 1. List of oligos representing the sequences of transcription factor binding site in CYP2C19 promoter region used for EMSA

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sequence of forward strand</th>
<th>Sequence of Reverse strand</th>
<th>Transcription factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’ CCA TCG TGG CGC ATT ATC TCT 3’</td>
<td>5’ AGA GAT AAT GCGCCA CGA GGT 3’</td>
<td>Cyclic AMP responsive element beta (CREB) binding site with normal allele in CYP2C19 promoter</td>
</tr>
<tr>
<td>2</td>
<td>5’ CCA TCG GGG CGC ATT ATC TCT 3’</td>
<td>5’ AGA GAT AAT GCCCGCCA TGG 3’</td>
<td>Cyclic AMP responsive element beta (CREB) binding site with variant allele in CYP2C19 promoter</td>
</tr>
<tr>
<td>3</td>
<td>5’ AAC ATT GTG CAA TTG 3’</td>
<td>5’ CAA TTG CAC AAT GTT 3’</td>
<td>CCAAT enhancing binding protein beta (CEBPB) binding site with normal allele in CYP2C19 promoter</td>
</tr>
<tr>
<td>4</td>
<td>5’ AAC ATT GGG CAA TTG 3’</td>
<td>5’ CAA TTG CCC AAT GTT 3’</td>
<td>CCAAT enhancing binding protein beta (CEBPB) binding site with variant allele in CYP2C19 promoter</td>
</tr>
</tbody>
</table>

3.3 Outcomes:
The outcomes of the work being carried out are as follows:

1. We observed differences in the interaction of normal and variant oligos representing the CREB and CEBPB binding sites with the nuclear protein in gel shift assays. This has been confirmed for CREB by competition experiments.
2. For both of these sites, a higher affinity of the nuclear protein was found with variant “mutated” oligos when compared to that of the most prevalent allele sequence, represented by oligos bearing the unmutated binding sites of the corresponding transcription factors.
3. Super shift assays with CREB-2 polyclonal antibody and CEBPB antibody revealed specific interaction with CREB but not with CEBPB.
4. This supports our earlier observations of increased luciferase activity with the constructs having variant allele in CREB binding site, suggesting the increased luciferase activity might be resulting from the increased interaction of CREB to its binding site in the presence of variant allele and thus increasing the transcription of the CYP2C19 gene.
5. This could explain how the variations in the cis acting elements in the promoter region of a gene could influences its transcription by altering the recruitment of the transcription factor to its binding site in a promoter region.
6. In silico docking analysis of homology modeled structure of CYP2C19 with its common substrates has revealed similar interaction of the substrates with inhibitory capacity with that of CYP2C19 (Table 2). Most of the substrates were docked into the hydrophobic pocket with residues such as ARG83, VAL25, PHE451, ALA272, ASP268, LEU341, GLY450, ILE219, GLU275, etc.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Substrate</th>
<th>Free energy from autodock*</th>
<th>Free energy from autodock vina*</th>
<th>H-bonds (donor-acceptor)*</th>
<th>Donor-acceptor distance</th>
<th>Donor-acceptor distance</th>
<th>Availability of Experimental/clinical reports on inhibitory activity on CYP2C19</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Omeprazole</td>
<td>-6.06</td>
<td>-8.3</td>
<td>#0 ARG 83 NE #1 LIG 1 O1 #0 ARG 83 HE</td>
<td>3.434</td>
<td>2.639</td>
<td>Reports are available on its inhibitory capacity</td>
</tr>
<tr>
<td>2</td>
<td>Rabeprazole</td>
<td>-5.62</td>
<td>-7.8</td>
<td>#0 ARG 83 NE #1 LIG 1 O #0 ARG 83 HE</td>
<td>3.208</td>
<td>2.326</td>
<td>Reports are available on its inhibitory capacity</td>
</tr>
<tr>
<td>3</td>
<td>Lansoprazole</td>
<td>-5.34</td>
<td>-8.7</td>
<td>#1 LIG 1 N #0 GLU 79 OE2 #1 LIG 1 H2</td>
<td>2.930</td>
<td>1.924</td>
<td>Contradictory reports are available</td>
</tr>
<tr>
<td>4</td>
<td>Pantoprazole</td>
<td>-4.55</td>
<td>-8.33</td>
<td>#1 LIG 1 N #0 GLY 79 O1 #1 LIG 1 H2</td>
<td>2.931</td>
<td>1.998</td>
<td>---</td>
</tr>
<tr>
<td>5</td>
<td>Clopidogrel</td>
<td>-3.97</td>
<td>-8.1</td>
<td>#0 ASN 179 ND2 #1 LIG 1 S #0 ASN 179 HD22</td>
<td>4.142</td>
<td>3.265</td>
<td>----</td>
</tr>
<tr>
<td>6</td>
<td>Voriconazole</td>
<td>-3.37</td>
<td>-8.4</td>
<td>#0 ARG 83 NE #1 LIG 1 N4 #0 ARG 83 HE</td>
<td>3.199</td>
<td>2.294</td>
<td>Reports are available on its inhibitory capacity</td>
</tr>
<tr>
<td>7</td>
<td>Gliclazide*</td>
<td>-4.48</td>
<td>-8.5</td>
<td>#0 ARG 83 NH2 #1 LIG 1 O1 #0 ARG 83 HH21</td>
<td>2.943</td>
<td>1.997</td>
<td>---</td>
</tr>
<tr>
<td>8</td>
<td>Glibenclamide*</td>
<td>-4.68</td>
<td>-10.8</td>
<td>#0 LYS 23 NZ #1 LIG 1 O2 #0 LYS 23 HZ2</td>
<td>3.316</td>
<td>2.446</td>
<td>---</td>
</tr>
<tr>
<td>9</td>
<td>Tolbutamide*</td>
<td>-3.04</td>
<td>-7.0</td>
<td>#1 LIG 1 N #0 GLU 380 OE1 #1 LIG 1 H6</td>
<td>2.900</td>
<td>1.906</td>
<td>----</td>
</tr>
</tbody>
</table>

* Similar clusters were found using the three different docking software i.e. Swissdock, autodock, and autodockvina. 
#0=target i.e. CYP2C19; #1=substrate i.e. corresponding substrate; Standard amino acid and atom symbols were used to represent the amino acid involved in interaction. Atoms are represented by their respective symbols (like ‘O’ for oxygen, ‘H’ for hydrogen, ‘N’ for nitrogen) followed by distance abbreviation in the order of increasing distance i.e. ‘A’ for alpha, ‘B’ for beta, ‘G’ for gamma, ‘D’ for delta, ‘E’ for epsilon, ‘Z’ for zeta, and ‘H’ for eta, followed by a digit designating the branch direction (No digit after distance abbreviation indicates that the side chain is unbranched). * Similar interaction was found between the family members except for gliclazide which is predicted to have some inhibitory capacity as it is interacting in a similar manner that of voriconazole and omeprazole. But till now no experimental or clinical reports are not available on its inhibitory capacity.
3.4 Future collaboration with host institution

We have established a contact with Prof. Nicolas Mermod group, LBTM, University of Lausanne and Prof. Oliver Michielin and Dr. Vincent Zoete of Swiss Institute of Bioinformatics. In future we may also plan some studies related to the gene regulation especially for those genes of therapeutic importance.

3.5 Various comments:

The host laboratory has organized my trip and schedule very well. The lab members and secretary were very cooperative. It was a pleasant and fruitful visit.

3.6 Projected publications/articles resulting or to result from the exchange

The findings of this project have to be communicated to a peer reviewed journal. After few more replicating experiments to confirm the results obtained, the manuscript with the data shall be communicated.