Chapter 4

Experimental Design
4.0 EXPERIMENTAL DESIGN

4.1 Need of the Present Study

Based on the literature, there is a much propensity for drug-drug interactions in patients with concurrent type 2 diabetes mellitus and HIV infection that are likely to be treated with antiretroviral and antidiabetic therapy.

Figure 4.1 Brief presentation of complexity associated with diabetic drugs and antiretroviral drugs

DDIs, Drug-drug interactions;
Most widely used PIs include indinavir, ritonavir and atazanavir, and NNRTIs include efavirenz and nevirapine.

Among oral hypoglycemic drugs, gliclazide is preferred in therapy because of its selective inhibitory activity towards pancreatic K⁺ATP channels, antioxidant property, low incidence of hypoglycemia and other haemobiological effects.¹⁹¹,¹⁹²

The complexity associated with the antidiabetic and antiretroviral drugs is shown in Figure 4.1.

However, there is no report/evidence on the effect of selected antiretroviral drugs (indinavir, ritonavir, atazanavir, efavirenz and nevirapine) on the pharmacodynamics and/or pharmacokinetics of gliclazide with respect to safety and efficacy of the combination which is crucial in clinical standpoint to provide rational therapy.

### 4.2 Objectives

**Primary Objectives:**

To investigate the safety and efficacy of the combination of selected antiretroviral drugs (indinavir, ritonavir, atazanavir, efavirenz and nevirapine) with gliclazide by conducting pharmacodynamic interaction studies in rats (normal and diabetic) and rabbits, and pharmacokinetic interactions studies in rabbits.

**Secondary Objectives:**

Information about the mechanism of interaction(s), if occurs.

The glucose disorders associated with PIs are either class specific or drug specific?
4.3 Animals Models

Drug interactions are usually seen in clinical practice and the mechanisms of interactions are evaluated usually in animal models (rodent and non-rodent). The normal rat model served to quickly identify the interaction and diabetic rat model served to validate the same response in the actually used condition of the drug.\textsuperscript{9,196,197} The rabbit model is another dissimilar species to validate the occurrence of the interaction. Usually, if the interaction is observed in rodent and non-rodent species, it is likely to occur in humans also, by considering their representative variability with humans.\textsuperscript{9,196,197} Although animal models can never replace the need for comprehensive studies in human subjects, their use can provide important insights to understand and to evaluate the potent interactions between drugs.\textsuperscript{179}

Cytochromes P450 are a superfamily of hemoproteins that play a central role in the metabolism of endogenous compounds and xenobiotics. Cytochrome P4503A isoforms (especially CYP3A4) are the most abundant human isoforms that metabolizes over one-half of clinically used drugs.\textsuperscript{235} However, it is well known that the metabolism of drugs may differ significantly between species, both in qualitative and quantitative terms.\textsuperscript{236} Nevertheless, with a careful selection of animal species and appropriate experimental design, it is possible to make a reasonably good prediction of the pharmacokinetics of drugs in humans. It is worth noting that several findings have confirmed the functional similarity of CYP isoforms in rabbits and humans, suggesting that the
rabbit is a valuable \textit{in vivo} model for the assessment of drug interaction occurring at the first pass of drugs ingested. Moreover, studies performed on rabbits, evaluating the pharmacokinetics of other drugs metabolized in humans \textit{via} CYP3A4 pathway have confirmed the usefulness of the rabbit model for such investigations. Based on these findings apart from convenience in serial blood sampling we preferred rabbit as animal model to perform the pharmacokinetic interaction studies. The multiple dose effect of antiretroviral drugs on the pharmacodynamics and pharmacokinetics of gliclazide in animal models were also studied since both are used for chronic period.

\textbf{Table 4.1 Description about experimental animals}

\begin{tabular}{|l|}
\hline
\textbf{Rodent Model: Albino Rats} \\
\hline
Provider & National Institute of Nutrition, Hyderabad \\
Sex & Both male and female \\
Age & 6 - 7 weeks \\
Weight & 250-350 g \\
\hline
\end{tabular}

\begin{tabular}{|l|}
\hline
\textbf{Non-rodent Model: Albino Rabbits} \\
\hline
Provider & National Institute of Nutrition, Hyderabad \\
Sex & Both male and female \\
Age & 3 - 5 months \\
Weight & 1.25 – 1.75 Kg \\
Commercial pellet diet & Rayan’s Biotechnologies Pvt Ltd., Hyderabad \\
\hline
\end{tabular}

- The animals were maintained under standard laboratory conditions at an ambient temperature of $25 \pm 2^\circ\text{C}$ and $50 \pm 15\%$ relative humidity with a 12:12 light/dark cycle.
- Animals were fed with a commercial pellet diet (Rayan’s Biotechnologies Pvt Ltd., Hyderabad, India) and water \textit{ad libitum}.
The animals were fasted for 18 hr prior to the experiment and during the experiment they were withdrawn from food and water.

- The animal experiments were performed after prior approval of the study protocol by the Institutional Animal Ethics Committee. The study was conducted in accordance with the guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

**Table 4.2 Description about experimental design**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Pharmacodynamic interaction studies between selected antiretroviral drugs with gliclazide in normal rats (N = 6)</td>
</tr>
<tr>
<td>II</td>
<td>Pharmacodynamic interaction studies between selected antiretroviral drugs with gliclazide in alloxan-induced diabetic rats (N = 6)</td>
</tr>
<tr>
<td>III</td>
<td>Pharmacodynamic and pharmacokinetic interaction studies between selected antiretroviral drugs with gliclazide in normal rabbits (N = 6)</td>
</tr>
</tbody>
</table>

**Note:** Pharmacodynamic, Pharmacokinetic and statistical methods were conducted as described in ‘Materials and Methods (Chapter 3)’ section.

The experimental design consists of three phases as shown in Table 4.2. Each phase contain five specific groups as shown below to conduct interaction study of 5 selected antiretroviral drugs with gliclazide.

Group I : Interaction study of indinavir with gliclazide (n = 6)
Group II : Interaction study of ritonavir with gliclazide (n = 6)
Group III : Interaction study of atazanavir with gliclazide (n = 6)
Group IV : Interaction study of efavirenz with gliclazide (n = 6)
Group V : Interaction study of nevirapine with gliclazide (n = 6)
The schematic representation of pharmacodynamic interaction studies and pharmacokinetic interaction studies in rats (normal and alloxan-induced diabetic) and rabbits was given in Figure 4.1 and Figure 4.2, respectively.

### 4.4 Pharmacodynamic Interaction Studies in Normal Rats

Each group of six rats was administered with gliclazide, orally. After one week washout period, the same group was administered with respective selected antiretroviral drug (indinavir/ritonavir/atazanavir/efavirenz/nevirapine) and after one week washout period the combination of interacting drug and gliclazide was administered. One week washout after this single dose interaction study, the respective group was continued with the daily treatment of selected respective antiretroviral drug (indinavir/ritonavir/atazanavir/efavirenz/nevirapine) for the next eight days with regular feeding. Later after 18 h fasting they were again given the combined treatment on the ninth day. Please refer Figure 4.2 for further information.

Blood was collected from the retro orbital plexus of each rat 0 (pre dose), 1, 2, 3, 4, 6, 8 and 12 hr for glucose estimation. Blood samples were collected from rats at 2 and 8 hr (time points where gliclazide has maximum activity in rats) for insulin estimation considering the limitation of the blood samples in rats. The primary pharmacodynamic parameters (glucose and insulin were estimated by GOD/POD method and radioimmunoassay methods, respectively) and secondary pharmacodynamic parameters (insulin resistance and $\beta$-cell function...
were determined by HOMA at 2 and 8 hr) as described in ‘materials and methods’ section.

4.5 Pharmacodynamic Interaction Studies in Alloxan-induced Diabetic Rats

Diabetes was induced in rats by the administration of alloxan monohydrate in two doses, i.e. 100 mg and 50 mg/kg bd. wt intraperitoneally for two consecutive days, as mentioned in materials and methods section. Rats with blood glucose levels of 200 mg/dL and above were considered as diabetic and selected for the study.

The same treatment (Single dose interaction study followed by multiple dose interaction study) as described in the study in normal rats was performed with a group of six alloxan-induced diabetic rats (Figure 4.2). The blood sample collection and estimation of pharmacodynamic parameters procedure is same as that of normal rats.

4.6 Pharmacodynamic and Pharmacokinetic Interaction Studies in Normal Rabbits

Each group of six rabbits was administered with gliclazide, orally. After one week washout period, the same group was administered with selected antiretroviral drug (indinavir/ ritonavir/ atazanavir/ efavirenz/ nevirapine) and after one week washout period the combination of interacting drug and gliclazide was administered. One week washout after this single dose interaction study, the respective group was continued with the daily treatment of selected respective antiretroviral
Figure 4.2 Pharmacodynamic Interaction Studies in Rats

Normal/alloxan-induced diabetic rats (N=6)

Stabilization

Gliclazide treatment

Washout period

ARVD treatment

Washout period

ARVD treatment, followed by gliclazide after 30 min

Washout period

ARVD drug treatment, up to 8 days with regular feeding

Combined treatment on 9th day after 18 hr fasting of the animals

Blood glucose level estimation at 0, 1, 2, 3, 4, 6, 8, 10 & 12 hr post dose by GOD/POD method

Insulin estimation by Radioimmunoassay method at 2 and 8 hr post dose

Determination of insulin resistance, and β-cell function by HOMA*

Statistical analysis using Student's paired t-test and interpretation of the data

ARVD, Antiretroviral drug; HOMA, Homeostasis model assessment

*Insulin resistance = (fasting serum insulin × fasting serum glucose)/22.5

*β-cell function = (20 × fasting serum insulin) / (fasting serum glucose − 3.5)
Figure 4.3 Pharmacodynamic and Pharmacokinetic Interaction Studies in Rabbits

Normal rabbits (N=6) → Stabilization → Gliclazide treatment → Washout period → ARVD treatment → Washout period → ARVD treatment, followed by gliclazide after 30 min → Washout period → ARVD drug treatment, up to 8 days with regular feeding → Combined treatment on 9th day after 18 hr fasting of the animals

Single dose pharmacodynamic and/or pharmacokinetic study

Blood glucose level estimation at 0, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20 & 24 hr post dose by GOD/POD method

Insulin estimation by Radioimmunoassay method at 1, 3, 8, 12, 16 & 24 hr post dose

Determination of insulin resistance, and β-cell function by HOMA*

Serum gliclazide estimation at 0 (pre dose), 1, 2, 3, 4, 6, 8, 10, 12, 16, 20 & 24 hr post dose by HPLC method

Pharmacokinetic analysis using WinNonlin software

Statistical analysis using Student’s paired t-test and interpretation of the data

ARVD, Antiretroviral drug; HOMA, Homeostasis model assessment

*Insulin resistance = (fasting serum insulin × fasting serum glucose)/22.5
*β-cell function = (20 × fasting serum insulin) / (fasting serum glucose − 3.5)
drug (indinavir/ ritonavir/ atazanavir/ efavirenz/ nevirapine) for the next eight days with regular feeding. Later after 18 h fasting they were again given the combined treatment on the ninth day. Please refer Figure 4.3 for further information.

Blood samples were collected from the marginal ear vein of each rabbit at 0 (pre dose), 1, 2, 3, 4, 6, 8, 12, 16, 20 and 24 h for glucose estimation. Blood samples were collected from rabbits at 1, 3, 8, 12, 16 and 24 hr for insulin estimation considering the feasibility and convenience of the blood samples in rabbits. The primary pharmacodynamic parameters (glucose by GOD/POD method and insulin by radioimmunoassay method) and secondary pharmacodynamic parameters (insulin resistance and β-cell function by HOMA at 1, 3, 8, 12, 16 and 24 hr) were determined as described in ‘materials and methods’ section.

Gliclazide concentration levels were estimated from rabbit serum by validated HPLC method as described in ‘materials and methods’ section. Pharmacokinetic analysis and statistical analysis were conducted as described in ‘materials and methods’ section.