IN VITRO EVALUATION

7.1 *In vitro* screening of EGFRTK Inhibition

Since the compounds were prepared using pharmacophore for kinase and EGFR TK inhibitors, and also docking results for EGFR TK inhibition were relatively good; it was thought to check the EGFR TK inhibition using *in vitro* assay. For studying the EGFRTK inhibitory potency of all the synthesized compounds, *in vitro* inhibition assay was done at BPS Biosciences, Santiago, USA. The concentration selected was 10µM as most of the compounds had IC_{50} value around 25 µM. Hence it was expected that at this concentration at least some inhibition would be seen.

7.2 ADP-Glo assay for Kinase activity

7.2.1 Principle

The ADP-Glo™ Kinase Assay is a luminescent ADP detection assay. It provides a homogeneous, high-throughput screening method to measure kinase activity by quantifying the amount of ADP produced during a kinase reaction. The assay can be used to monitor the activity of virtually any ADP-generating enzyme (e.g., kinase or ATPase) using up to 1mM ATP. The assay is performed in two steps; first, after the kinase reaction, an equal volume of ADP-Glo™ Reagent is added to terminate the kinase reaction and deplete the remaining ATP. Second, the Kinase Detection Reagent is added which converts ADP to ATP and allows the newly synthesized ATP to be measured using a luciferase/luciferin reaction.

Luminescence in luminometer can be correlated to ADP concentrations. This assay is sensitive enough to detect very low amounts of ADP (20nM). The luminescent signal generated is proportional to the ADP concentration produced and is correlated with kinase activity [91].

The ADP-Glo™ Kinase assay relies on the properties of a thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to generate a stable “glow-type” luminescent signal based on ADP-ATP conversion.

The assay consists of two step, first step consists of enzymatic reaction which results in ADP generation, thus ATP and ADP Both are present in the well. Further ADP-Glo™ Reagent addition results in elimination of the remaining ATP. In the second step kinase detection reagent is added to convert the remaining ADP into ATP. This reagent also contains everything needed to measure the newly generated ATP with the help of a luciferase/luciferin reaction. The

luminescence measured after incubation is proportional to the ADP concentration generated during the enzymatic reaction.

Kinase inhibitor when added in kinase reaction it reduces or inhibits the conversion of ATP to ADP. When all ATP are removed using ADP-Glo™ reagent, the reduced concentration of ADP produces less luminescence.

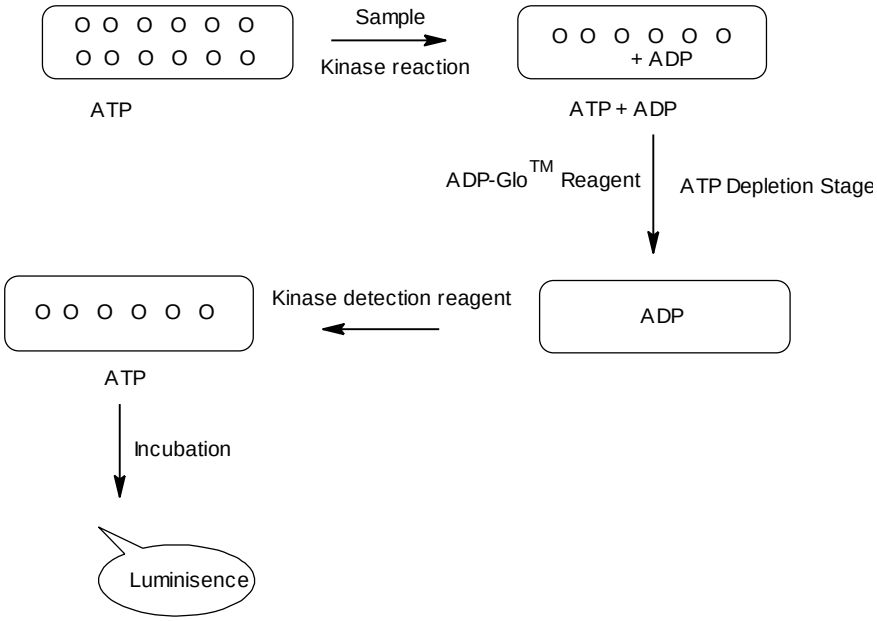

Figure 7.1 . Principle of the ADP-Glo™ Kinase Assay.

7.2.2 Methodology

The all compounds were diluted in 10% DMSO and 5µl of the dilution was added to a 50µl reaction to get the final concentration of DMSO i.e. 1% in all of reactions.

All of the enzymatic reactions were conducted at 30^{o}C for 30 min. The 50 µl reaction mixture contains 40 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 1 mM DTT, 0.2 mg/ml Poly (Glu, Tyr) substrate, 10 µM ATP and EGFR. For the 30 min preincubation, the enzyme was preincubated with the inhibitor for 30 min at room temperature and the reaction was initiated by adding the ATP and substrate solution. After the enzymatic reaction, 50 µl of Kinase-Glo Plus luminescence kinase assay solution (Promega) was added to each reaction and incubated the plate for 15 min at room temperature. Luminescence signal was measured using a BioTek Synergy 2 microplate reader.

Table 7.1: Materials for kinase assay

Substrate	0.2 mg/ml Poly (Glu, Tyr), 10 µM ATP
Assay kit	Kinase-Glo Plus Luminescence kinase assay kit (Promega#V3772)
Standard	Staurosporine (a known kinase inhibitor)

7.3 Results and discussion

When the compounds were tested against EGFRTK using kit, all the compounds showed poor inhibition. The readings were not significantly different than that of vehicle control. Although docking results for maximum compounds were promising, in vitro assay results were not in concurrence.