Screening and characterization of novel inhibitors of mitotic kinesin Eg5 targeting new allosteric pocket

ATP Driven Motor Protein Research Group

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1. Background and Aim of Study

It is a kinesin ATP-driven motor domain protein moving along the microtubules. eg-5 is a kinesin family member which has an important physiological role related to mitosis. Eg5 is important because of its vital role in mitosis since It has been found that eg5 is Highly expressed in cancer cells. if we can specifically inhibit eg5, we can overcome cancer by reaching apoptosis and arresting cell cycle. Therefore, Eg5 has been considered as a target for cancer therapy, and inhibitors specific for Eg5, such as Monastrol, and STLC, have been developed. Interestingly, they target the same binding pocket Allosteric site (AS1) composed of helix α2, α3 and loop L5, located around 10 Å away from the ATPase site. In this research we focus on targeting the binding pocket (AS2) composed of α4, α6 located around 15 Å away from the ATPase site. To achieve this aim we collaborate with the AI pharmaceutical Company Atomwise Inc. Thus, our laboratory has received 96 chemical compounds from ATOMWISE. The compounds were subjected to *in vitro* screening experiments against the basal &MT stimulated ATPase activity of Eg5 to obtain the potent inhibitors which are potential candidates for AS2.

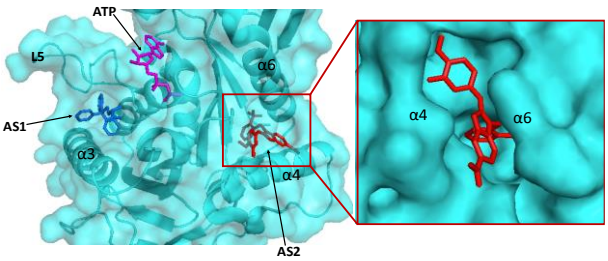

Fig.1 3D Structure of kinesin Eg5 showing the binding sites.

2. Materials and Methods

Preparation of kinesin Eg5 Recombinant Eg5 was prepared as previously described (Ishikawa *et al*., 2014). Briefly, cDNA of mouse Eg5 motor domain (WT, residues 1-367) was amplified by polymerase chain reaction and ligated into the pET21a vector. The Eg5 WT expression plasmids were adopted to transform *Escherichia coli* BL21 (DE3). The Eg5 WT was then purified with a Co-nitrilotriacetic acid column which was washed with lysis buffer containing 30mM imidazole. Bound Eg5 was eluted with lysis buffer containing 150 mM imidazole. The obtained fractions were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) whereas dialysis of purified Eg5 was done using a buffer containing 30 mM Tris-HCl [pH 7.5], 120 mM NaCl, 2 mM $MgCl_2$, 0.1 mM ATP and 0.5 mM DTT. Finally, the purified Eg5 was stored at -80°C till for further experiments.

ATPase assay:

Biomol method: ATPase activity was measured at 25°C in ATPase assay solution containing Tris-HCl (pH 7.5), $MgCl_2$, NaCl and distilled water. 10µM or 100µM inhibitor, dissolved in DMF, was added, and followed by 0.5 µM kinesin Eg5. Total volume of assay solution was 500 µl which was vortexed well and left to incubate at 25°C for 5 minutes before the addition of 1mM ATP. The ATPase activity was terminated after 15 minutes by addition 10% TCA. The inorganic phosphate (Pi) released in the reaction was estimated spectrophotometrically (96 well plates in a microplate reader apparatus) using the Biomol green reagent at 630nm.

2. RESULTS and DISCUSSION

General screening was carried at 10 **µ**M in both types; basal (Fig. 2) and microtubules (Fig. 3), then the dose was raised up to 100 **µ**M for the best selected compounds, Compound 32 displayed the highest modulatory effect (Fig. 4) to show later a dissociation constant (kd) of 50µM and a saturation at 100µM without completely inhibiting the ATP hydrolysis function of Eg5 (Fig. 5).

2-1. Basal screening results

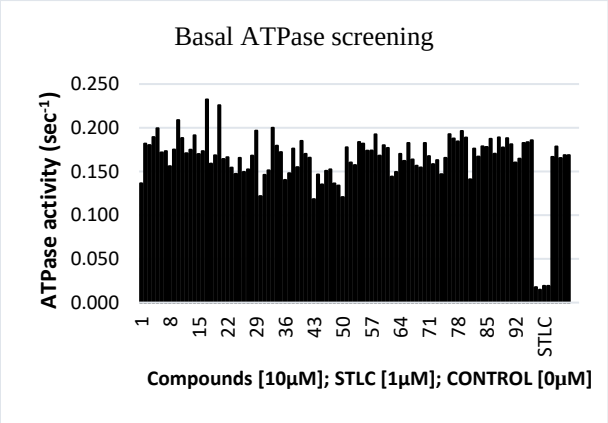

Fig.2. ATP concentration was 1mM, Eg5 concentration was 0.5 µM and incubated for 15 minutes.

2-2. MT stimulated screening results

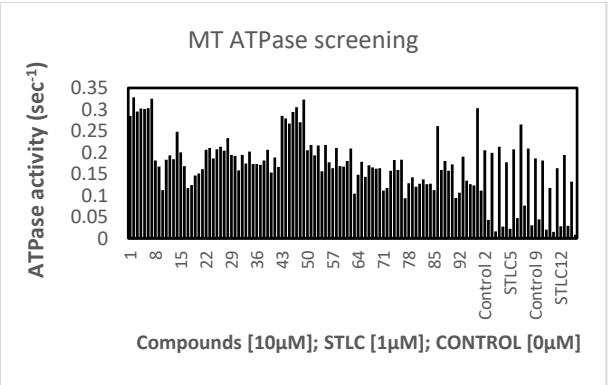

Fig. 3. Screening of compounds at concentration of ATP was 1mM, Eg5 was 0.1, Microtubule was 3 µM and incubated for 5 minutes.

2-3. Inhibition of ATPase Activity of Selected compounds

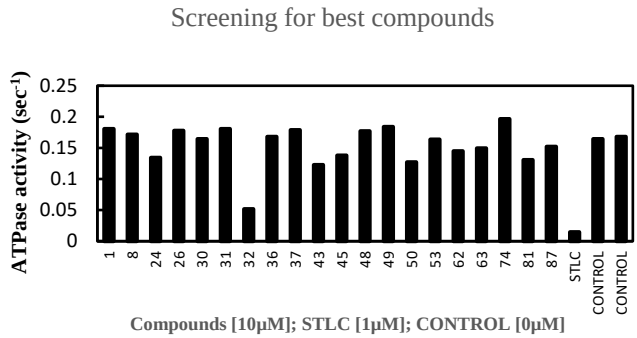

Fig. 4. Screening at 100µM of inhibitors. concentration of ATP was 1mM, Eg5 was 0.5 µM.

2-3. IC50 value of Best compound (#32):

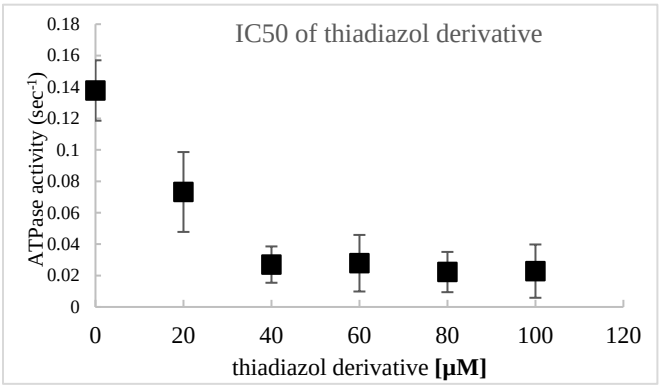

Fig. 5. Dose dependent for (#32), concentration of ATP was 1mM, Eg5 was 0.5 µM and incubated 15min.

CONCLUSION

'thiadiazol derivative' displayed the highest effect with a IC50 of 50µM and a saturation at 100µM without completely inhibiting the ATP hydrolysis function of Eg5.

Compared to STLC at 1 µM, the effect of 'thiadiazol derivative' was very moderate.

Future Plans

1. Measure the motility Assay for the best two compounds.
2. Kinetic study using stopped-flow apparatus.

References

1. Ishikawa, K., Tohyama, K., Mitsuhashi, S., and Maruta, S. (2014) Photocontrol of the mitotic kinesin Eg5 using a novel S-trityl-L-cysteine analogue as a photochromic inhibitor. *J. Biochem.* **155**, 257–263.