がん治療の標的である有糸分裂キネシンEg5の新規機能性阻害剤の開発

Development of novel functional inhibitor for mitotic kinesin Eg5 as a target cancer therapy

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SYNOPSIS

キネシン Eg5 は、微小管をレールとして駆動する ATP 駆動型のモーター蛋白である。細胞周期の M 期の進行において Eg5 は細胞有糸分裂に関わる重要な役割を担っている生体分子機械である。Minstrel 、STLC、Ispinesib などの特異的阻害剤は、Eg5 による有糸分裂を停止させて、ガン細胞の増殖を抑えることによりアポトーシスを誘導することが示されており、抗ガン剤として注目されている。またその阻害の分子機構は明らかにされており、Eg5 の制御ナノデバイスとして捉えることができる。先行研究において、これまでにアゾベンゼンなどのフォトクロミック分子の誘導体が、これらの阻害剤を模倣して光可逆的に Eg5 の機能を阻害することが明らかにされている。本研究では、異なる 2 つのフォトクロミック分子であるアゾベンゼンとスピロピランの誘導体を融合させた、これまでに無い高効率、且つ多段階の光スイッチ機構をもつフォトクロミック Eg5 阻害剤の開発を行い、細胞レベルで Eg5 の活性を光可逆的に制御することを試みた。そして 3 段階で Eg5 の機能を制御できることを示した。この方法により細胞機能の多段階の光制御および機能性抗ガン剤への応用が期待される。

「Keywords」Motor protein, Kinesin Eg5, Photochromic Compounds, Eg5 inhibitors & Isomerization.

1. Introduction

Kinesin is a motor protein that plays an important role in animal and plant cells. It hydrolyses ATP and moves along microtubules. It means that motor proteins are extremely fine machines that can efficiently convert the chemical energy into mechanical energy by ATP hydrolysis. In fact, martin et.al. propose that motor proteins could be applied to various fields as “nanomachines” [(1)]. Kinesin Eg5 also known as Kinesin spindle protein (KSP) and it belongs to the motor protein. It is involved in the transport of the endoplasmic reticulum and organelles, and in the transport of neurotransmitters from nerve bodies to synaptic terminals in nerve axons. It also plays a crucial role in mitotic cell division for the formation of the bipolar spindle in the eukaryotic cell division [(2)]. Therefore, it has been suggested that mitotic kinesin Eg5 is considered as a potential target for cancer therapy.

The various functions of Eg5 are proposed in equivalent polymorphisms in the key kinesin structural elements. One of the functional elements of Eg5 is referred to as Loop 5 (L5). L5 of Eg5 has a very long structure unlike other kinesins and acts as the target for binding of small molecular inhibitors. For the application of Eg5 motor proteins as nanomachines, it is essential to artificially control the mechanical structure that changes with external stimulation. Interestingly, several small-molecule compounds (such as monastrol, S-Triyl-L-Cysteine (STLC), Ispinesib, and so on have been shown the Eg5 inhibitory activity. They are binding to the same druggable Eg5 allosteric pocket, which is composed of Loop L5, α2, and α3. However, they showed structural diversity and controlled the activity of Eg5 unidirectionally or irreversibly and as a result, they could not be applied as nanodevice to control the functional activity of Eg5 reversibly. Therefore, switching is required not only to control Eg5 activity in reverse but also to control functional activity.

To do that I focused on chromism. Chromism refers to a phenomenon in which the optical properties (color, fluorescence, etc.) of a substance are reversibly changed by external stimulation such as heat, pH, light, and so on. A substance that exhibits chromism is called a chromic substance (or chromic material). First, it is suggested that to establish a switchable nanodevice, heat could be worked like as- high, low, and room temperature. However, in this study, I have used a protein that is highly sensitive to high temperatures. Therefore, thermal is not an appropriate technique for switching. Subsequently, I focused on the development of switching with different pH such as- acidic, alkaline, and neutral pH. Unfortunately, both the acidic and alkaline pH are harmful to the general structure of the protein. Hence, pH could not be applicable for working as a switching. Finally, I focused on light for the establishment of switching. It has been well known that photochromic compounds show light sensitivity. Photochromic compounds are those compounds that show their structural and functional changes depends upon the light irritation. There are two types of mechanisms observed for returning to their original states. A mechanism that returns by irradiating light with a different wavelength, it’s called P-type such as diarylethene and fulgide. Another one, a mechanism that returns by heat, it’s called T-type such as spiropyran, azobenzene, and stilbenes. Interestingly, some of the photochromic compounds show structural similarity with well-known potent Eg5 inhibitors such as spiropyran. Sadakane, et.al, designed and synthesized a novel photochromic Eg5 inhibitor composed of double photochromic compounds, spiropyran, and azobenzene [(3)]. The inhibitor displayed two isomerization states and controlled Eg5 activity. Generally, one photochromic compound shows two isomerization states as shown in Fig.1.

Theoretically, coupling of two different photochromic compounds could show multiple isomerization states which would be effective to establish photo switching precisely.

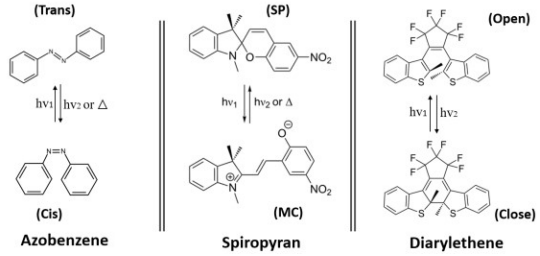

Fig. 1. List of photochromic compounds and their isomer forms

In our laboratory, photo switching has been used as a well-established technique to control protein activity for the last two decades. I focused on obtaining novel inhibitors from synthetic or natural sources and then react with photochromic compounds. The most changeling task is to design of photochromic inhibitor for controlling Eg5 activity in multiple stages. In this study, Firstly, I designed and combined two different photochromic compounds to obtain inhibitory activity in multiple states such as SPSAB (spiropyran and azobenzene). Secondly, I identified a new and potent Eg5 inhibitor from natural sources. Eg5 inhibitor from natural sources that I utilized in this research is Kolaroflavon as shown in Fig.2. and it exhibited effective control action for Eg5. In the future, that could be formed multiple states with photochromic compounds (e.g- STLC- Azobenzene derivatives, Ishikawa et.al).

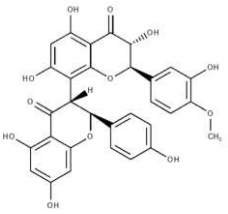

Fig. 2. A new natural source potent Eg5 inhibitors.

The aim of the study, the development, and formation of multiple states of functional Eg5 inhibitors, which have clinical significance in the treatment of cancer patients. The inhibitory activity of drugs could be controlled precisely, in terms of high dosage, low dosage, or medium dosage, considering the patient's condition. The aim of the study, the formation of multiple states is of clinical significance in the treatment of cancer patients. The inhibitory activity of drugs could be controlled precisely, in terms of high dosage, low dosage, or medium dosage, considering the patient's condition.

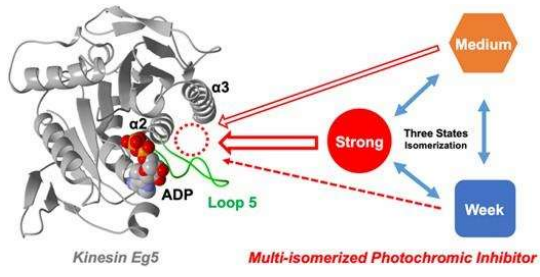

Fig. 3. Schematic representation of the proposed multiple photoregulation by the novel photochromic inhibitor which exhibits three isomerization states.

2. Designed and combined of two different photochromic compounds to synthesis SPSAB

I employed a photochromic compound combination of azobenzene and spiropyran to create a photochromic inhibitor that exhibits multiple inhibitory activities for kinesin Eg5. Previously, we had shown that the homodimer of azobenzene or spiropyran derivatives is a potent photochromic inhibitor. Therefore, the heterodimers of azobenzene and spiropyran are also expected to show inhibitory activity. SP-COOH was coupled with 4-aminoazobenzene-4'- sulphonic acid according to the established methods of our laboratory. The incorporation of sulphonic acid is expected to increase solubility in physiological solutions. Azobenzene isomerizes between cis and trans, and spiropyran exhibits spiro and merocyanine isomers. Therefore, the SPSAB composed of azobenzene and spiropyran can theoretically form four isomerization states, SP-trans, SP-cis, MC-cis and MC-trans (Fig. 4).

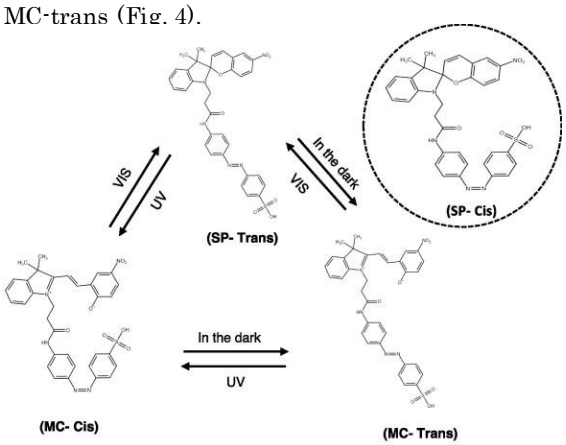

Fig. 4. Photoisomerization of the spiropyran-sulphonated azobenzene (SPSAB).

2.1. Photocontrol of the SPSAB inhibitory activity on the basal and MTs stimulated Eg5 ATPase

The inhibitory activity of SPSAB in the three isomers states of SP-trans (VIS), MC-cis (UV) and MC-trans (in the dark) were examined on the basal ATPase activity of Eg5 without MTs. As shown in Fig. 5A, the three SPSAB isomers inhibited basal ATPase activity in a concentration-dependent manner with different inhibitory activities. The half-maximal inhibitory concentrations (IC_{50}) for the three isomerization states were estimated. The SP-trans isomer showed the highest inhibitory activity at an IC_{50} value of 30μM. In contrast, MC-cis had the lowest IC_{50} value of 86.0 μM among the three isomers. The IC_{50} of MC-trans was 38.7 μM.

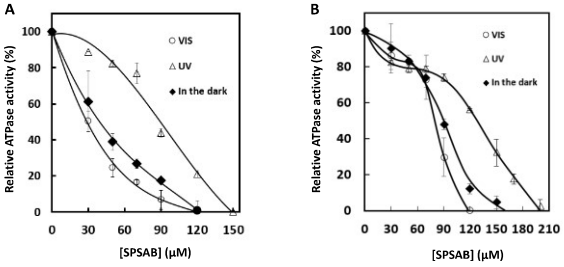

Fig. 5. Photoregulation of Kinesin Eg5 ATPase using three-state isomerization of SPSAB.

Subsequently, I also examined the inhibitory activity of SPSAB on MT-stimulated ATPase activity among the three states. As shown in Fig. 5B, MT-stimulated ATPase activity was also inhibited by the three SPSAB isomers in a concentration-dependent manner with different inhibitory activities for each isomer. The influence of SPSAB on the interaction between Eg5 and MTs was also examined. I measured the MT concentration-dependent ATPase activity of Eg5 in the presence of the three isomerization states of SPSAB. The V_{max} values of MC-cis and MC-trans were almost the same, 60% of V_{max} in the absence of SPSAB. SP-trans showed a slightly higher V_{max} than the other two states, as shown in Table I. On the other hand, the three isomers clearly exhibited different K_{MT} (Table I).

Table I. Calculation of V_{max} and K_{MT} values of Eg5 ATPase in the presence of three isomerization states of SPSAB

Inhibitors (isomerization)	V_{max} (s^{-1})	K_{MT} (μM)
Control (DMF)	10.24 ± 0.36	1.58 ± 0.2
MC-*cis* (UV)	6.63 ± 0.6	2.53 ± 0.6
MC-*trans* (in the dark)	6.78 ± 1.97	4.51 ± 2.25
SP-*trans* (VIS)	7.1 ± 0.86	5.9 ± 0.65

2.2. Photoregulation of Eg5 motor activity with SPSAB

An in vitro motility assay using fluorescently labelled MTs was performed according to established methods of our laboratory. The gliding velocity of the MTs on the Eg5 adsorbed glass surface in flow cells was measured in the presence of the three isomers. In the absence of the SPSAB isomer (control), the average MT gliding velocity was 10.78±1.47 nm/s, as shown in Fig. 6A. In the presence of the SP-trans isomer, the average velocity of MTs clearly slowed down to 8.47±1.1 nm/s (Fig. 6B).

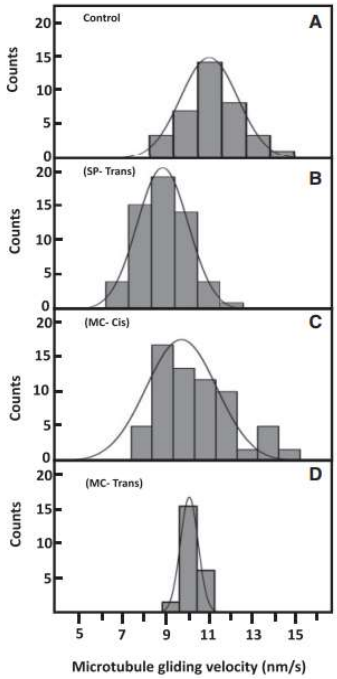

Fig. 6. Observation of the effect of SPSAB on Eg5 motor activity using fluorescence microscopy in vitro motility assay.

The histogram of the velocity in the presence of MC-cis showed a slightly broader distribution range from control to SP-trans, with the MTs gliding average velocity being 10.01±1.78nm/s, as shown in Fig.6C. The MC-trans isomer showed an intermediate average velocity of 9.56±0.5 nm/s compared with those of the other two isomers in Fig. 6D.

3. The potent Eg5 inhibitor from natural sources which could be formed multiple states with photochromic compounds

Some synthetic small molecule inhibitors of the protein, such as monastrol, S-trityl-L-cysteine (STLC) and ispinesib, have been reported. However, the natural sources of Eg5 inhibitors remain relatively less explored. Only a few Eg5 inhibitors from natural products, including gossypol, terpendol E and adociasulfate-2, are known. Medicinal plants are an indispensable part of natural products that are continually being explored for their beneficial therapeutic effects and bioactive components. Among such plants, Garcinia species are famous for their potent biological activities in various experimental models. A yellowish extract termed 'kolaviron' has been isolated from the nuts of Garcinia kola as the predominant phenolic constituent. Interestingly, the active compounds in kolaviron have been identified as biflavonoids, namely Garcinia biflavonoid (GB) 1, GB 2 and kolaflavanone (KLF), which are implicated in kolaviron-associated bioactivities. However, the biochemical analysis of KLF interactions with Eg5 has not been performed. Hence, I investigated the possible effect of KLF on Eg5 ATPase and motility activities. Such an effect may be responsible, at least in part, for the anticancer potential of KLF. The data presented in this study demonstrate the allosteric inhibition of Eg5 ATPase and microtubule-gliding activity by KLF, as well as the possible mechanisms of Eg5–KLF interaction. In future, it could be a candidate for the formation of multiple states with photochromic compounds.

3.1. KLF inhibits Eg5 ATPase activity

To ascertain the potential of KLF to interact with kinesin Eg5 and inhibit its function, the basal ATPase activity was measured in the absence of microtubules at various concentrations (0–250 mM) of KLF. As shown in Fig. 7A, KLF inhibited the basal ATPase function of Eg5 in a dose-dependent manner with an IC_{50} value of 98 μM. Next, we evaluated the effect of KLF on the microtubule-activated ATPase activity of Eg5.

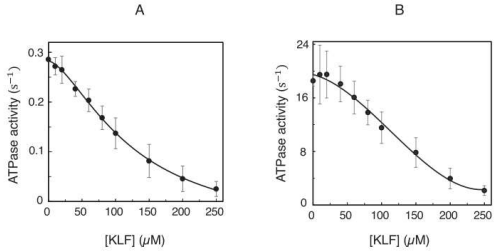

Fig. 7. Effect of KLF on the in vitro ATPase activity of Eg5.

The results revealed a concentration-dependent inhibition with an IC_{50} value of 125 μM (Fig. 7B). The inhibitory activity of KLF on the basal ATPase activity of Eg5 was more potent than that of microtubule activated activity, indicating that microtubules moderate the inhibitory potency of KLF.

3.2. Interaction mechanism between kinesin Eg5 and KLF

To gain more insights into the interaction between KLF and Eg5, including the possible binding site on the motor domain of Eg5, we investigated whether KLF competes with ATP at the nucleotide-binding pocket. The concentration of ATP was increased from 0 to 250μM in the absence of microtubules, whereas the concentration of KLF was fixed at 100μM, which was chosen based on its IC_{50} in the basal ATPase activity. As shown in Fig. 8A, KLF does not directly compete with ATP binding at the nucleotide-binding (active) site. The V_{max} for ATP was reduced from 3.37±0.37 s^{-1} in the absence of KLF to 2.47±0.55 s^{-1} in the presence of 100μM KLF. However, the K_m for ATP (35.77μM) in the absence of KLF was very similar to that at 100μM KLF (35.23μM). Indeed, the K_m of ATP obtained for Eg5 in this study is compatible with that reported by Cochran et al. and Lad et al. These data suggest that KLF inhibits Eg5 via a non-competitive mechanism rather than a competitive mechanism in which an inhibitor is expected to increase K_m without altering the V_{max}. Similarly, we evaluated the ATPase activity of Eg5 in the presence of 100μM KLF while varying the microtubule concentration from 0 to 12μM (Fig. 8B). The K_{MT} obtained for microtubule association of Eg5 in the absence of KLF was 0.98μM, and the V_{max} was 31.55 s^{-1}. However, the value of KMT was increased to 2μM in the presence of 100μM KLF, whereas that of V_{max} was decreased to 28.38 s^{-1}.

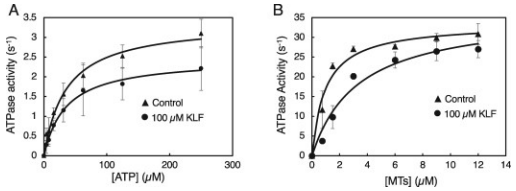

Fig. 8. Effect of KLF on the MT-dependent Eg5 ATPase activity.

3.3. Specificity of inhibitory effect of KLF against kinesin Eg5

The inhibitory effect of KLF was mapped out on conventional kinesin (kinesin 1) to evaluate whether the inhibitory effect of KLF is specific to Eg5 among the kinesin superfamily. As shown in Fig.9, KLF suppressed the microtubule-activated ATPase activity of kinesin-1 (22%) and Eg5 (62%) at 150 μM. The effect was dose-dependent, suggesting that the inhibition may be caused by the binding of KLF to a similar site on both Eg5 and conventional kinesin. However, the affinity of KLF for kinesin-1 was lower than that for Eg5. Previous crystallographic and computational studies of Eg5 and kinesin-1 have shown that the loop5 is more elongated in Eg5 than in conventional kinesin. Since this loop contributes to the formation of the allosteric binding pocket of Eg5, it may explain the weaker binding affinity of KLF for kinesin 1.

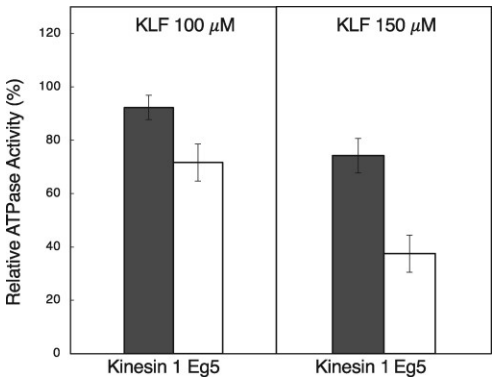

Fig.9. Inhibitory effect of KLF on the ATPase activity of conventional kinesin

4. Discussion

The three isomerization states of SPSAB successfully exhibited different inhibitory activities. The inhibitory activity of SPSAB of the SP-trans isomer for the basal and MT-dependent ATPase activity of Eg5 exhibited potent IC_{50} values, whereas MC-trans and MC-cis exhibited medium and weak IC_{50} values. Therefore, this study proved that photochromic molecules are valid as photoswitching devices to control the inhibitory activity of the mitotic kinesin Eg5 inhibitor. The experimental data also suggest that the natural sources a new and potent Eg5 inhibitors KLF inhibited the ATPase activity of Eg5 in basal and microtubule-activated states. KLF allosterically inhibited the ATPase activity and microtubule-gliding function, without directly competing with ATP and microtubule binding.

5. Conclusion

The novel photoresponsive kinesin Eg5 inhibitor, SPSAB, forms three isomerization states: SP-trans (VIS), MC-cis (UV) and MC-trans (in the dark). The photochromic inhibitor SPSAB significantly affected the activity of Eg5. Most importantly, the formation of three states was achieved to control Eg5 precisely. It is expected that the three states of SPSAB may provide more options for the treatment of cancer.

Another natural compound KLF exhibited the evidence of the Eg5 inhibitory function. That is an important achievement for the development of anticancer drug. In future, it would be the candidate to form multiple states with combination of photochromic compounds.

6. References

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