Analysis of the Effect of Glaziovianin A and the Derivatives on Microtubule Dynamics

September 2014

Takumi CHINEN

Analysis of the Effect of Glaziovianin A and the Derivatives on Microtubule Dynamics

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Agricultural Science

(Doctoral Program in Life Sciences and Bioengineering)

Takumi CHINEN

Introduction

Microtubules, a component of the cytoskeleton in eukaryotes, have a cylindrical structure that consists of polymerized α/β-tubulin. Microtubules are involved in spindle formation in mitosis, cellular signaling, cell motility and the determination of organelle positions.

In mitosis, there is a key regulation machinery, mitotic/spindle checkpoint which regulate the entry to anaphase. In this checkpoint system, the kinetochore in the prophase/early prometaphase recruits several mitotic checkpoint components, (e. g. BUBR1, MAD2, CEMPE and more), which inhibit the CDC20-dependent recognition of cyclin B and securin by Anaphase promoting complex/Cyclosome (APC/C). When the microtubules are captured and tension is detected at the kinetochore, the mitotic checkpoint is satisfied, and the cell cycle progresses with entries into anaphase by the degradation of cyclin B and securin by APC/C.[1] But when even one chromosome is not properly captured by microtubules, the mitotic checkpoint is activated to keep cells from aneuploidy and apoptotic cell death. Because cancer cells have abnormal mitotic checkpoints, miss segregation and apoptosis are easily induced in cancer cells.[2]

Microtubule polymerization inhibitors (e.g., *vinca* alkaloids) and microtubule

stabilizers (e.g., taxane) have been used for cancer chemotherapy. These microtubule inhibitors prevent spindle formation and arrest cell cycle progression at M-phase by activating the mitotic/spindle checkpoint, subsequently inducing cell death in cancer.[3] However, these microtubule inhibitors can also cause some severe side effects, including peripheral neuropathy. It is thought that this side effect occurs due to the extreme effects on the microtubules of non-proliferative cells, which are in interphase.[3]

Against this background, microtubule dynamics have been recognized as an attractive target for cancer chemotherapy that may help avoid the side effects of microtubule destabilizers and stabilizers. Microtubule plus ends repeatedly grow and then shorten, and this phenomenon is called "microtubule dynamics". These dynamics are regulated by GTP/GDP on β-tubulin.[4] In α-tubulin, GTP is not hydrolyzed and it supports the structure of tubulin heterodimer. In contrast, GTP bound to β-tubulin is hydrolyzed, and the hydrolysis reaction changes the conformation of the α/β-tubulin heterodimer, and subsequently the de-polymerization of microtubule occurs (this transition forward to de-polymerization is called a "catastrophe"). Microtubule dynamics consist of three phases, growing, shortening, and pause (neither growing nor shortening) and two transitions: catastrophe (transition from growing or pause to shortening) and rescue (transition from shortening to growing or pause).

For example, the microtubule dynamics inhibitor, eribulin, a synthetic analog of halicondrine B,[5] is marketed by Esai Pharmaceutical Company and used to treat patients with metastatic breast cancer who have received at least two prior treatments in the EU,[6] U.S.[7] and Japan.[8] This compound binds to the plus end of microtubules and inhibits microtubule elongation but not shortening.[9,10] Another microtubule dynamics inhibitor, noscapin, is undergoing a phase I/II clinical trial for multiple myeloma.[3] These compounds have little effect on the microtubule network in interphase cells, but they induce abnormal spindle formation. Except for their interference with bipolar spindle formation, the effects of microtubule dynamics inhibitors on the cell functions have not been revealed. It is thus quite important to understand the functions of microtubule dynamics in cells to prevent the side effects induced by microtubule dynamics inhibitors.

Glaziovianin A (AG1, Fig. 1), a new isoflavone isolated from the leaves of Brazilian pea *Ateleia glazioviana*, induces abnormal spindle formation (an abnormal alignment of chromosomes and multi-polar spindles) and arrests cell cycle progression of mammalian cells at M-phase.[11] In a cancer cells panel screening performed by the Screening Committee for Anticancer Drugs (SCAD), AG1 showed similar score to those of microtubule polymerization inhibitors,[11] suggesting that AG1 is a microtubule

polymerization inhibitor. In previous studies, the effects of AG1 was investigated on the microtubules and it was revealed that AG1 delayed tubulin polymerization *in vitro* (Fig. 2) and that the signals of EB1 (a microtubule plus end binding protein), which accumulates at growing microtubule plus ends,[12, 13] disappeared soon after AG1 treatment (Fig. 3). These data suggested that AG1 directly binds tubulin heterodimer and attenuated microtubule polymerization, and probably de-polymerization as well. However, the details of the effects of AG1 on microtubules and its inhibitory mechanisms remain to be revealed. In this thesis, therefore, I investigated the binding site of AG1 on tubulin heterodimer and AG1's effect on microtubule dynamics *in vitro* and *in vivo*.

To analyze the mode of action of small molecules, not only mammalian cells[14,15] but also a model organism such as yeast[16,17] are often used. Mammalian cells are useful for analyses, such as observation of organelles using microscopy,[14,15], biochemical experiments,[15] because they are easy to observe their organelles morphology and to lyse the cells. In the field of chemical biology, mammalian cell's high sensitivities against small compounds facilitates the analyses of mode of action. However, analyses of microtubule inhibitors are sometimes difficult because human cells have approximately eight α- and eight β-tubulin isoforms. Tubulins function as heterodimer, the number of

the combination of α- and β-tubulin isoforms would thus be more than 60. In addition, several posttranslational modifications of microtubules, such as acetylation, phosphorylation, polyglutamylation and polyglycylation, have been reported.[18] These complexity make analysis of microtubule inhibitors difficult.

The budding yeast *Saccharomyces cerevisiae*, is also a useful tool for chemical biology studies because it is easy to use genetic analyses, mutational analyses,[16] gene disruption, and genome modification[14,17,19] (Fig. 4). *S. cerevisiae* has only two α-tubulin genes (*TUB1* and *TUB3*) and one ß-tubulin gene (*TUB2*), and *TUB3* gene can be disrupted.[20] *S. cerevisiae* can thus express single allele of α-/β-tubulin isoform (Tub1p and Tub2p) and genetic modifications of these genes are useful ways to analyze the mode of action of AG1 and other microtubule inhibitors.[21] However, there is a major obstacle for mode of action analyses using *S. cerevisiae*. Budding yeast is resistant to most of the compounds to which mammalian cells are sensitive (Table 1). Therefore, new strains showing high sensitivities to AG1 are required for the analysis of the mode of action of AG1.

In our laboratory, the molecular targets or binding sites of several bioactive compounds were determined using yeast genetics.[14,16,17] To determine the target molecules and/or the binding site, it has been necessary to construct yeast strains

suitable for the various compounds,[16,22,23] but the construction of deletion strains that have sensitivities specific to each of the various compounds is a time-consuming process. Hence I speculated that the deletion of all of these genes (except those that are important for viability and genetic experiments) could increase the drug sensitivity without influencing transformation, mating, or sporulation efficiency.

There are two factors conferring multidrug resistance in *S. cerevisiae*: one is the drug efflux system, and other is the permeability barrier. The drug efflux system is composed of ATP-binding cassette (ABC) transporters that export xenotoxic compounds to outside of cells or inside of vacuole[24-26] and the transcriptional factors of ABC transporters.[25,26] *S. cerevisiae* has at least 16 ABC transporters, of which Pdr5p, Snq2p and Yor1p confer multidrug resistance by exporting bioactive small molecules out of cells. And four transcriptional factors (Pdr1p, Pdr3p, Pdr8p and Yrr1p) up-regulate the transcription of most of the ABC transporters. In contrast, the major factor for the permeability barrier is ergosterol in the yeast plasma membrane. For these reasons, multidrug-sensitive strain lacking *PDR1*, *PDR3* (transcriptional factors for ABC transporters) and *ERG6* (ergosterol synthase) are used for drug screening.[27] However, this deletion mutant which lacks 3 genes (*pdr1*, *pdr3*, and *erg6*) decreases the transformation and sporulation efficiencies that are essential for yeast genetic analysis. I

therefore hypothesized that it is possible to make highly multidrug-sensitive strain by the disruption of ABC transporters.

In this thesis I focused on ABC transporters on only the plasma membrane (including Pdr5p, Snq2p and Yor1p) and their transcription factors. The constructed strain lacking eight ABC transporters and four transcription factors showed sensitivity against several compounds, but they were not sensitive to AG1 or AG1 derivatives. I therefore next introduced the *ERG6* inducible expression system, and finally succeeded in constructing a strain showing sensitivity to AG1 derivative.

Abstract

In this thesis, I analyzed the effect of AG1 and the derivatives on the MT dynamics, and constructed multidrug hypersensitive yeast strain 12geneΔ0HSR-iERG6 for investigation of the inhibitory mechanism of AG1.

The MT dynamics analysis using EGFP-α-tubulin-expressing PtK2 cells revealed that AG1 decreases both of elongation and shortening velocities, and the frequency of catastrophes. These changes of parameter drastically increased the population of MT in pause phase, without depolymerization as described previously. Because it was revealed that AG1 inhibits MT dynamics of which the function is still unclear, therefore the function of MT dynamics was analyzed using AG1. My analysis revealed that AG1 inhibited EGF-stimulated EGFR transport without changing MT network. This data suggest that MT dynamics in cells regulate the endosome transport. These findings indicate that AG1 will be a useful tool to investigate the function of MT dynamics in cells.

AG1 binds colchicine site on tubulin heterodimer, and it is expected that the analysis of the binding mode of AG1 in more detail will give new knowledge on the regulation of MT dynamics by inhibitors. However, human cells have several tubulin isoforms and posttranslational modifications of MTs, these complexity make

biochemical analysis of MT inhibitors difficult. On the other hand, *S. cerevisiae* can express single allele of α-/β-tubulin isoform and genetic modifications of these genes are useful ways to analyze AG1. However, *S. cerevisiae* is resistant to most of the compounds including AG1, therefore multidrug-sensitive strain 12geneΔ0HSR-iERG6 was constructed for analyzing the details of the mechanism of MT dynamics inhibition by AG1. 12geneΔ0HSR-iERG6 showed improved drug sensitivity to compounds including O^7-propargyl AG1. Furthermore this strain showed sufficient transformation and sporulation efficiencies under the galactose condition, which are important property for genetic approach. The results suggest that 12geneΔ0HSR-iERG6 is useful tool to analyze not only O^7-propargyl AG1 but also other small molecules using genetics approach.

References

1) Kops, G. J., Weaver, B. A., and Cleve, D. W. (2005) On the road to cancer: aneuploidy and the mitotic checkpoint. *Nat. Rev. Cancer*. 5, 773-785.

2) Jackson, J.R., Patrick, D.R., Dar, M. M., and Huang, P.S. (2007) Targeted anti-mitotic therapies: can we improve on tubulin agents? *Nat. Rev. Cancer* 7, 107-117.

3) Dumontet, C., and Jordan, M. A. (2010) Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nat. Rev. Drug Des*. 9, 790-803.

4) Howard, J., and Hyman, A. A. (2009) Growth, fluctuation and switching at microtubule plus ends. *Nat. Rev. Mol. Cell Biol.* 10, 569-574.

5) Towle, M. J., Salvato, K. A., Budrow, J., Wels, B. F., Kuznetsov, G., Aalfs, K. K., Welsh, S., Zheng, W., Seletsky, B. M., Palme, M. H., Habgood, G. J., Singer, L. A., Dipietro, L. V., Wang, Y., Chen, J. J., Quincy, D. A., Davis, A., Yoshimatsu, K., Kishi, Y., Yu, M. J., and Littlefield, B. A. (2001) In vitro and in vivo anticancer activities of synthetic macrocyclic ketone analogues of halichondrin B. *Cancer Res*. 61, 1013−1021.

6) Pean, E., Klaar, S., Berglund, E. G., Salmonson, T., Borregaard, J., Hofland,

K. F., Ersbøll, J., Abadie, E., Giuliani, R., and Pignatti, F. (2012) The European medicines agency review of eribulin for the treatment of patients with locally advanced or metastatic breast cancer: summary of the scientific assessment of the committee for medicinal products for human use. *Clin Cancer Res.* 18, 4491-4497.

7) U.S. Food and Drug Administration on HALAVEN™ (eribulin mesylate) Injection. U.S. Food and Drug Administration. http://www.access-data.fda.gov/drugsatfda_docs/label/2010/201532lbl.pdf. Accessed January 4th, 2013.

8) Hattori, M., Fujita, T., Sawaki, M., Kondo, N., Horio, A., Ushio, A., Gondo, N., Idota, A., Ichikawa, M., and Iwata, H. (2013) [Clinical efficacy and safety assessment of eribulin monotherapy in patients with metastatic breast cancer - a single- institute experience] *Gan To Kagaku Ryoho* 40, 737-741.

9) Jordan, M. A., Kamath, K., Manna, T., Okouneva, T., Miller, H. P., Davis, C., Littlefield, B. A., and Wilson, L. (2005) The primary antimitotic mechanism of action of the synthetic halichondrin E7389 is suppression of microtubule growth. *Mol. Cancer Ther.* 4, 1086−1095.

10) Smith, J. A., Wilson, L., Azarenko, O., Zhu, X., Lewis, B. M., Littlefield, B.

A., and Jordan, M. A. (2010) Eribulin binds at microtubule ends to a single site on tubulin to suppress dynamic instability. *Biochemistry* 49, 1331−1337.

11) Yokosuka, A., Haraguchi, M., Usui, T., Kazami, S., Osada, H., Yamori, T., and Mimaki, Y. (2007) Glaziovianin A, a new isoflavone, from the leaves of Ateleia glazioviana and its cytotoxic activity against human cancer cells. *Bioorg. Med. Chem. Lett.* 17, 3091-3094.

12) Schuyler, S. C. and Pellman, D. (2001) Microtubule "plus-end-tracking proteins": The end is just the beginning. *Cell* 105, 421-424.

13) Salaycik, K. J., Fragerstrom, C. J., Murthy K., Tulu U. S., and Wadsworth P. (2005) Quantification of microtubule nucleation, growth and dynamics in wound-edge cells. *J. Cell Sci.* 118, 4113-4122.

14) Usui, T., Kazami, S., Dohmae, N., Mashimo, Y., Kondo, H., Tsuda, M., Terasaki, G. A., Ohashi, K., Kobayashi, J., and Osada, H. (2004) Amphidinolide H, a potent cytotoxic macrolide, covalently binds on actin subdomain 4 and stabilizes actin filament. *Chem. Biol.* 11, 1269-1277.

15) Chinen, T., Kazami, S., Nagumo, Y., Hayakawa, I., Ikedo, A., Takagi, M., Yokosuka, A., Imamoto, N., Mimaki, Y., Kigoshi, H., Osada, H., and Usui, T. (2013) Glaziovianin A prevents endosome maturation via inhibiting

microtubule dynamics. *ACS Chem Biol.* 8, 884-889.

16) Miyamoto, Y., Machida, K., Mizunuma, M., Emoto, Y., Sato, N., Miyahara, K., Hirata, D., Usui, T., Takahashi, H., Osada, H., and Miyakawa, T. (2002) Identification of Saccharomyces cerevisiae isoleucyl-tRNA synthetase as a target of the G1-specific inhibitor Reveromycin A. *J. Biol. Chem.* 277, 28810-28814.

17) Kazami, S., Muroi, M., Kawatani, M., Kubota, T., Usui, T., Kobayashi, J., and Osada, H. (2006) Iejimalides shows anti-osteoclast activity via V-ATPase inhibition. *Biosci. Biotechnol. Biochem.* 70, 1364-1370.

18) Janke C., and Bulinski, J. C. (2011) Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. *Nat. Rev. Mol. Cell Biol.* 12, 773-786.

19) Li, J., Katiyar, S. K., and Edlind, T. D. (1996) Site-directed mutagenesis of Saccharomyces cerevisiae beta-tubulin: interaction between residue 167 and benzimidazole compounds. *FEBS Lett.* 385, 7-10.

20) Schatz, P. J., Solomon, F., and Botstein, D., (1986) Genetically essential and nonessential alpha-tubulin genes specify functionally interchangeable proteins. *Mol. Cell Biol.*, 6, 3722-3733.

21) Gupta, M. L. Jr, Bode, C. J., Georg, G. I., and Himes, R. H. (2003) Understanding tubulin-Taxol interactions: mutations that impart Taxol binding to yeast tubulin. *Proc. Natl. Acad. Sci. U S A.* 100, 6394-6397.

22) Foland, T. B., Dentler, W. L., Suprenant, K. A., Gupta, M. L. Jr, and Himes, R. H. (2005) Paclitaxel-induced microtubule stabilization causes mitotic block and apoptotic-like cell death in a paclitaxel-sensitive strain of Saccharomyces cerevisiae. *Yeast* 22, 971-978.

23) Arita, Y., Nishimura, S., Matsuyama, A., Yashiroda, Y., Usui, T., Boone, C., and Yoshida, M. (2011) Microarray-based target identification using drug hypersensitive fission yeast expressing ORFeome. *Mol. Biosyst.* 7, 1463-1472.

24) Decottignies, A., and Goffeau, A. (1997) Complete inventory of the yeast ABC proteins. *Nat. Genet.* 15, 137-145.

25) Bauer, B. E., Wolfger, H., and Kuchker, K. (1997) Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. *Biochim. Biophys. Acta.* 1461, 217-236.

26) Jungwirth, H., and Kuchler, K. (2006) Yeast ABC transporters-- a tale of sex,

stress, drugs and aging. *FEBS Lett.* 580, 1131-1138.

27) Simon, J. A., and Bedalov, A. (2004) Yeast as a model system for anticancer drug discovery. *Nat. Rev. Cancer* 4, 481-492.