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Application of Bioprobes for Mammalian Cell Cycle Analyses

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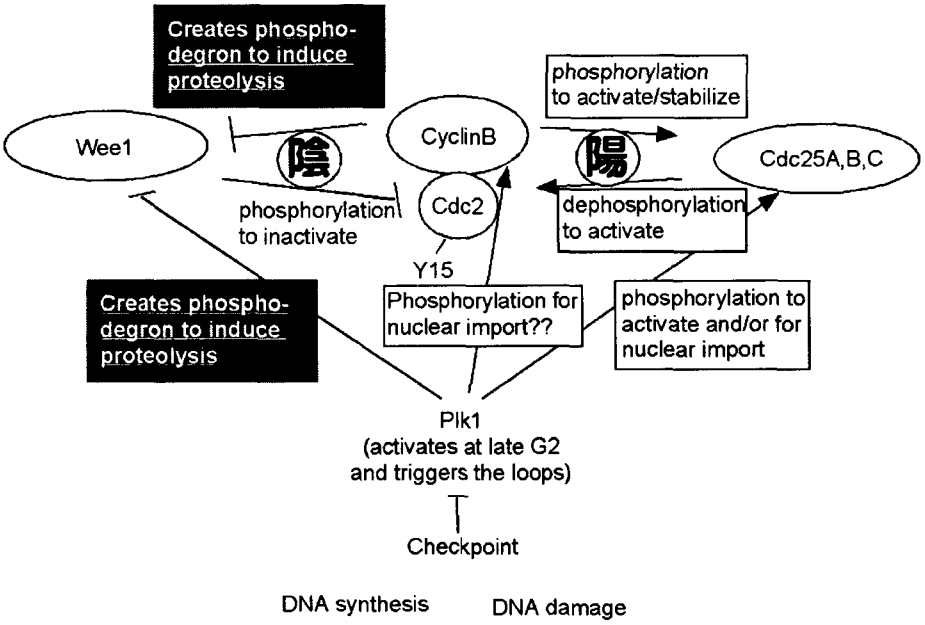
Introduction

Systematic screening of the microbial metabolites beyond antibiotics has yielded many kinds of the biologically active substances, such as immunosuppressants, antitumor agents, etchas been. Nowadays, microbial metabolites are recognized as a treasure box containing new biological activities as well as novel chemical structures. In this presentation, I would like to introduce our research activity on development of "terminology bioprobe", which is generally reserved for biochemical reagent (1). Bioprobes are not only useful for investigating cell functions but also not only possible candidates for as medicines (2).

Control mechanism of m-phase promotion

In mammalian cells, disorder of cell cycle control causes many pathological phenomena, such as tumor. Revealing the mechanism of the cell cycle regulation is a good way for developing novel antitumor agents (3). Recently, we have revealed a control mechanism of Cdc2 kinase which is essential for cell division (4). Cdc2 kinase is inactivated in the interphase through negative phosphorylation by two protein kinases, Wee1 and Myt1. When cells are ready for mitosis, the Cdc25 phosphatase activates Cdc2 by removing inhibitory phosphates at Thr14 and Tyr15. Activated Cdc2 phosphorylates Cdc25 in turn, increasing its activity. This positive feedback loop ensures rapid activation of Cdc2 at the onset of mitosis. At the same time, Myt1 is inactivated by phosphorylation, while Wee1 is inactivated both by phosphorylation and degradation. However, the feedback loop between Cdc2 itself and Wee1/Myt1 in somatic cells has not been elucidated, since phosphorylation of Wee1 and Myt1 by Cdc2 did not affect their activity. We show that phosphorylation of human somatic Wee1 by Cdc2, and a second M-phase kinase Plk1 creates phospho-degrons (signals for degradation) for the β-TrCP1/2 F-box protein-containing SCF E3 ubiquitin ligase ($SCF^{\beta\text{-}TrCP1/2}$) thereby inducing proteasome-dependent degradation that is required for the initiation of mitosis (Fig. 1). Our results establish the existence of a feedback loop between Cdc2 and Wee1 in somatic cells that is dependent on ubiquitination and protein degradation.

Fig. 1. Positive and negative feedback loops for the activation of Cdc2 upon the onset of mitosis



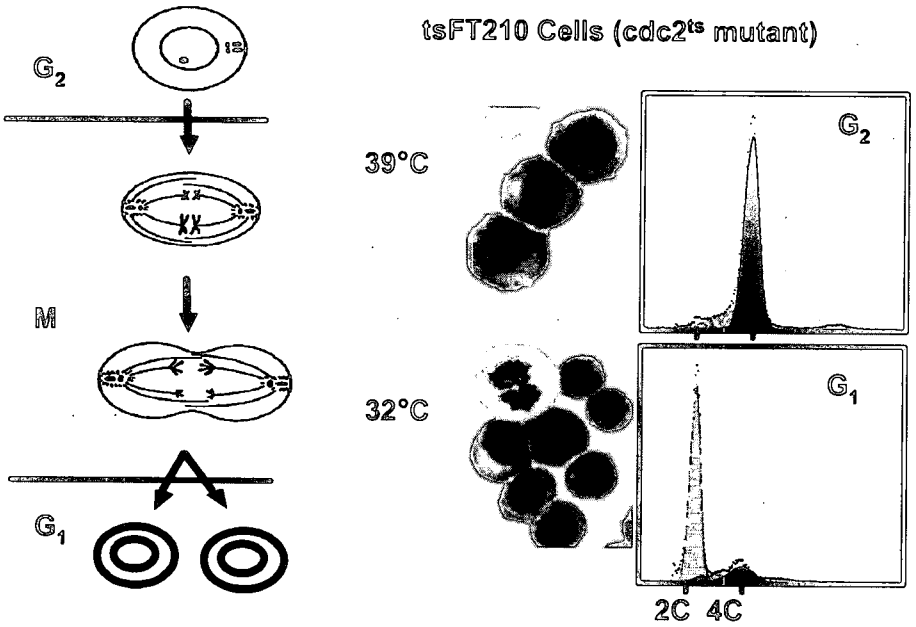
M-phase inhibitors

Drugs that interact with microtubules are expected to be useful not only as antitumor agents, but also as tools for understanding a wide variety of the cellular functions of microtubules, such as mitosis, cell signaling, and motility in eukaryotes. Microtubule inhibitors displaying great structural diversity have been identified and well characterized because of their extensive application in medicinal and basic research. Paclitaxel and vinblastine are clinically used as anticancer drugs. They bind to different sites of β-tubulin and show opposite effects *in vitro*; paclitaxel induces microtubule bundling and vinblastine induces microtubule disassembly. We have reported that tryprostatin A inhibited microtubule-associated protein-dependent tubulin assembly by binding to tubulin at binding sites different from those used by colchicine and vinblastine (5). However, in most of the cases, microtubule-directed drugs bound to β -tubulin with a few exceptions; dinitroaniline herbicides and B-ring of colchicine.

Pironetin

We have screened new M-phase inhibitors from microbial metabolites using the following bioassay system (Fig. 2). tsFT210 cells are a defect in cdc2 kinase, which is a component of M-phase promoting factor. Cultured at high temperature, the kinase activity is destroyed and the cell cycle arrested at the G2 phase. When the temperature is shifted down to 32°C, the cell cycle arrest can be released from the G2 phase. About 4-hours later, most of the cells pass the M-phase and enters in the G1 phase. This bioassay system is highly sensitive to detect the microbial extract containing the M-phase inhibitors(6).

Fig. 2. Bioassay System for Cell Cycle Inhibitors

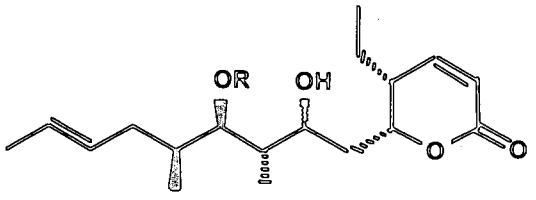


During the screening, we have identified pironetin as an M-phase inhibitor. Pironetin disrupted the tubulin network but no effect on the actin cables in fibroblasts. We have isolated three derivatives of pironetin from the same producing strain (Fig. 3). Pironetin has the most potent compound among them. In CDF1 mice bearing p388 leukemia cells, pironetin showed a moderate antitumor effect (7).

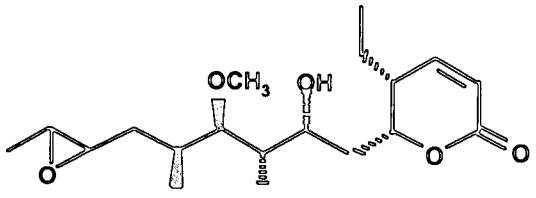
Pironetin inhibits tubulin binding of radiolabeled vinblastine, and the affinity of pironetin to tubulin is stronger than that of vinblastine (8). Furthermore, pironetin has a unique structure containing only one pyran residue and an alkyl chain, which is simpler than the structure of other M-phase inhibitors. These features of pironetin, which are distinct from known tubulin-binding agents, suggest that it is possible to create a new drug useful for cancer therapy from pironetin as a lead compound. From the analyses of structure-activity relationships, it was revealed that α, β-unsaturated lactone is important for microtubule inhibition (9). This characteristic strongly suggests that pironetin covalently binds to tubulin by Michael addition.

We determined the binding site of pironetin to be the Lys352 of α-tubulin by using biotinylated analogs as probes. Pironetin is the first compound shown to bind to α -tubulin directly. The binding site is located on the surface of α-tubulin facing the β-tubulin of the next heterodimer, which corresponds to the vinblastine-binding site (10).

Fig. 3. Pironetin Derivatives



Pironetin : R = CH_3
Demethylpironetin : R = H



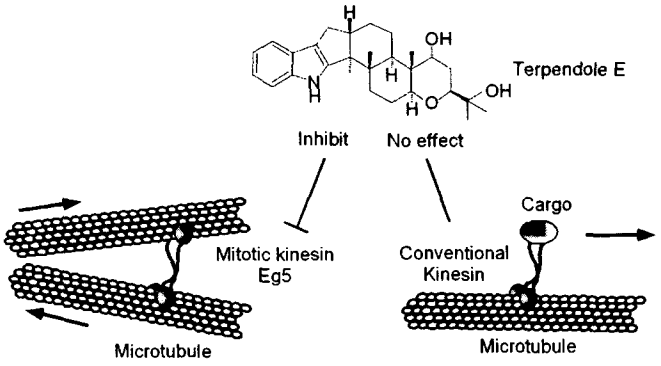
Epoxypironetin

Terpendole E

To reveal the mechanism of mitosis, the development of M-phase specific inhibitors is an important strategy. Haggarty et al. developed the cytoblot assay in order to screen for drugs that block the progression of the cell cycle specifically in the M-phase (11). Using this assay, drugs which do not directly interact with microtubules but which interact with microtubule-associated proteins and kinesins were discovered. A newly synthesized compound, named monastrol, has been shown to inhibit Eg5, a member of the kinesin family, and it induces the formation of monoaster spindles; however, it does not inhibit microtubule polymerization directly (12).

We have been screening microbial products to find the specific M-phase inhibitors without direct interaction to tubulins, and re-discovered terpendole E as a novel Eg5 specific inhibitor(13). Terpendole E did not affect the microtubule integrity in the interphase, but induced the formation of a monoastral spindle in M phase (Fig. 4). Terpendole E inhibited both the motor and microtubule-stimulated ATPase activities of the human Eg5, but did not affect the conventional kinesins. Although terpendoles were reported as an acyl-CoA:cholesterol *O*-acyltransferase (ACAT) inhibitor(14), the Eg5 inhibitory activity of terpendole E was independent of ACAT inhibition.

Fig. 4. Terpendole E inhibits Eg5 activity



Perspective

Technological The technology innovation is necessary to create more systematic and integrative methods for new drug discovery. The genome research is providing information on telling us the structure and function of proteins that are responsible for diseases. It will be possible to make a specific bioprobe to bind to a the specific protein. Such a bioprobe should be a good candidate for a tailor-made- medicine, which can be applied to a is served for each patient with a particular different genetic background. "Chemical Biology" using bioprobes as well as structural biology focusing on functional protein function will should be one of the big sciences in for the twenty-first century.

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