Molecular Pharmacology Fast Forward. Published on November 14, 2006 as doi:10.1124/mol.106.029611

MOL#29611

Nordihydroguaiaretic Acid Affects Multiple Dynein-dynactin Functions in Interphase and Mitotic Cells

Kohei Arasaki, Katsuko Tani, Tamotsu Yoshimori, David J. Stephens, and Mitsuo Tagaya

School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan (K.A., K.T., M.T.); Department of Cellular Regulation, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan (T.Y.); and Department of Biochemistry, University of Bristol, School of Medical Science, University Walk, Bristol, United Kingdom (D.J.S.)

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Effect of NDGA on dynein-dynactin

Address correspondence to: Mitsuo Tagaya, School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan. Tel: +81-42-676-5419; Fax: +81-42-676-5468; E-mail: tagaya@ls.toyaku.ac.jp.

Number of Text Pages: 28

Number of Tables: 0

Number of Figures: 6

Number of References: 40

Number of Words: Abstract (215 words); Introduction (531 words); Discussion (1039 words)

ABBREVIATIONS: NDGA, nordihydroguaiaretic acid; ER, endoplasmic reticulum; MT, microtubule; Ab, antibody; IC, intermediate chain; Noc, nocodazole; DMSO, dimethyl sulfoxide; Tf, transferrin; RNAi, RNA interference; TfR, transferrin receptor.

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Nordihydroguaiaretic acid (NDGA), a well-known lipoxygenase inhibitor, has actually pleiotropic effects on cells, which include cell proliferation, apoptosis, differentiation, and chemotaxis. We and others have previously shown that this compound causes Golgi disassembly by an unknown mechanism. Here we show that, in parallel with Golgi disassembly, NDGA induces the accumulation of the microtubule minus-end-directed motor dynein-dynactin complex at the centrosome, where microtubules minus-ends lie. Concomitant with this accumulation, dynein-dynactin-interacting proteins, such as ZW10 and EB1, were also redistributed to the centrosomal region. In cells where microtubules were depolymerized by nocodazole, NDGA promoted the formation of filaments consisting of dynein-dynactin and its interacting proteins, suggesting that it stimulates the association of these proteins in an ordered, not random, manner. Loss of dynactin function abolished not only NDGA-induced redistribution in intact cells, but also filament formation in nocodazole-treated cells. The latter finding implies that dynactin is a key molecule for the association between dynein-dynactin and its interacting proteins. In mitotic cells, NDGA induced robust accumulation of dynein-dynactin and its interacting proteins at the spindle poles. These results taken together suggest that NDGA perturbs membrane traffic by affecting the function of the microtubule motor dynein-dynactin complex and its auxiliary proteins. To our knowledge, NDGA is the first case of a reagent that can modulate dynein-dynactin-related processes.

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

(Introduction)

Nordihydroguaiaretic acid (NDGA) is a drug that affects a wide variety of cellular processes, including growth factor- and tumor necrosis factor-induced signal transduction (Domin et al., 1994; Lee et al., 2003; West et al., 2004), leukocyte chemotaxis (Goetzl, 1980), myoblast cell differentiation (Ito et al., 2005), cancer cell proliferation (Avis et al., 1996; McDonald et al., 2001; Seufferlein et al., 2002; Youngren et al., 2005), and viral proliferation in infected cells (Gnabre et al., 1995). It can also induce nitric oxide synthase expression (Ramasamy et al., 1999), regulate calcium channel activity (Korn and Horn, 1990; Huang et al., 2004), and inhibit growth of β-amyloid (1-40) protofibril (Moss et al., 2004). Although many of the effects of NDGA on cellular events appear to be ascribable to its action as a lipoxygenase inhibitor or an antioxidant, some are obviously peculiar to the action of NDGA (Korn and Horn, 1990; Lee et al., 2003; Huang et al., 2004; Ito et al., 2005). The anti-cancer activity of NDGA may be in part due to its inhibition of protein kinase C and receptor tyrosine kinases (Domin et al., 1994; Youngren et al., 2005).

We have shown for the first time that NDGA perturbs intracellular membrane traffic. NDGA inhibits the vesicle-mediated transport of vesicular stomatitis virus-encoded glycoprotein both within the Golgi apparatus in vitro (Tagaya et al., 1993) and from the endoplasmic reticulum (ER) to the Golgi in vivo (Tagaya et al., 1996). Later studies demonstrated that NDGA induces Golgi disassembly (Yamaguchi et al., 1997), which leads Golgi components to be redistributed to the ER (Drecktrah et al., 1998; Fujiwara et al., 1998a). In addition to the secretory pathway, this

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

compound blocks the endocytic pathway in human dendric cells in a manner independent of inhibition of lipoxygenases and prevention of reactive oxygen species formation (Ramoner et al., 1998), raising the possibility that NDGA affects the machinery generally required for vesicular transport, but not that for specific transport processes. In this context, the finding of Nakamura et al. (2003) that showed that NDGA is capable of stabilizing microtubules (MTs) is worth noting because they generally participate in membrane transport along the secretory and endocytic pathways. However, the relationship between the two NDGA-induced effects, MT stabilization and transport defect, is totally unknown.

We have recently demonstrated that, in interphase cells, ZW10 is present in the ER membrane, as well as in the cytosol, and plays a role in membrane traffic between the ER and Golgi (Hirose et al., 2004). ZW10 was originally characterized as a kinetochore-associated component that interacts with dynamitin (Starr et al., 1998), a subunit of dynactin that provides a link between the MT minus-end-directed motor dynein and cargo molecules. In the course of our study on ZW10, we found that NDGA induces the accumulation of ZW10 at the centrosome, where MT minus-ends lie. To better understand the mechanism for NDGA-induced Golgi disassembly, we examined in detail how NDGA induces ZW10 redistribution. We found that NDGA affects dynein-dynactin such that this motor associates more tightly with ZW10. The enhanced association may allow a long-range movement of dynein-dynactin and its interacting proteins toward the centrosome, which leads to imbalance in membrane traffic, thereby causing Golgi disassembly.

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Antibodies (Abs). Monoclonal Abs against dynamitin, EB1 and $p150^{Glued}$ were obtained from Transduction Laboratory. Monoclonal Abs against dynein intermediate chain (IC) and γ-tubulin were purchased from Santa Cruz Biotechnology and Accurate Chemical and Scientific Corp., respectively. The preparation and sources of other Abs were described previously (Yoshimori et al., 1988; Hirose et al., 2004).

Chemicals. Ascorbic acid, *N*-acetyl cysteine, α-tocopherol, propidium iodide, and taxol were obtained from Wako Pure Chemicals. Nocodazole (Noc) and 5, 8, 11, 14-eicosatetraynoic acid were obtained from Sigma. NDGA was purchased from Biomol Research Lab and freshly dissolved in dimethyl sulfoxide (DMSO) before use. When cells were incubated with these reagents, fetal calf serum was omitted from culture medium. Transferrin (Tf)-FITC was purchased from Molecular Probe.

Plasmid, Cell Culture, and Transfection. The full-length cDNA of dynamitin was inserted into pFLAG-CMV2. HeLa cells were cultured in Eagle's minimum essential medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin and 10% fetal calf serum. Transfection of cells with plasmids was performed according to the manufacturer's protocol using LipofectAMINE PLUS reagent (Invitrogen). Double thymidine block was performed as described by Chan et al. (2000).

Immunoprecipitation and Immunofluorescence. Preparation of cell lysates and immunoprecipitation were performed as described previously (Hirose et al., 2004).

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

Immunofluorescence microscopy was performed as described (Hirose et al., 2004). Unless otherwise stated, cells were fixed with methanol –20°C for 5 min.

Semi-intact Cells. To express ZW10-GFP, Tet-on inducible ZW10-GFP expression cells (Hirose et al., 2004) were incubated with 1.0 μg/ml doxycycline for 48 h. The cells were washed twice with permeabilization buffer (25 mM Hepes-KOH (pH 7.4), 115 mM potassium acetate, 2.5 mM $MgCl_2$, 2 mM EGTA and 1 mg/ml glucose), and then permeabilized with 40 μg/ml digitonin in the same buffer at 0°C for 5 min. After washing with permeabilization buffer twice, the cells were incubated in 1 ml of the reaction mixture at 32°C for 1 h. The reaction mixture contained permeabilization buffer plus an ATP-regenerating system (8 mM creatine phosphate, 1 mM ATP and 50 μg/ml creatine kinase) and 2.5 mg/ml rat liver cytosol.

RNA Interference (RNAi). Duplex RNAs for targeting were ZW10 (102) (5'-AAGGGTGAGGTGTGCAATATG-3') and $p150^{Glued}$ (207) (5'-TGATGGAACTGTTCAAGGC-3'). They were purchased from Japan Bioservice. RNAi experiments were conducted as described previously (Hirose et al., 2004)

Uptake and Recycling of Tf. Uptake and recycling experiments were conducted as described (Hirose et al., 2004) with a slight modification.

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

Results

NDGA Causes Redistribution of ZW10 to the Centrosomal Region. Upon incubation of HeLa cells with 30 μM NDGA for 1 h, ZW10 was redistributed from the ER to the perinuclear area in parallel with dispersion of a Golgi marker protein, GM130 (Fig. 1A, +NDGA, top and middle rows). The perinuclear region where ZW10 was accumulated was marked by a centrosome marker, γ-tubulin (bottom row).

To gain insight into the mechanism for NDGA-induced ZW10 redistribution, we first examined whether this redistribution is caused by scavenging of reactive oxygen species or inhibiting lipoxygenases. Cells were incubated with an antioxidant (α-tocopherol, ascorbic acid, or *N*-acetyl cysteine) or a lipoxygenase inhibitor (5, 8, 11, 14-eicosatetraynoic acid) for a prolonged time, and then the distribution of ZW10 was analyzed. As shown in Fig. 1B, none of the reagents induced the redistribution of ZW10 to the centrosomal region. In addition, taxol, a well-known MT-stabilizing reagent, did not cause ZW10 redistribution. These results indicate that ZW10 redistribution to the centrosomal region is not due to the prevention of reactive oxygen species formation, inhibition of lipoxygenases, or stabilization of MTs.

To determine whether energy and/or cytosolic factors are necessary for NDGA-induced ZW10 redistribution, we used cells expressing ZW10-GFP. ZW10-GFP-expressing cells were permeabilized to remove the cytosol (including cytosolic ZW10-GFP), and then NDGA, together with ATP and rat liver cytosol, was added. As shown in Fig. 1C, ZW10 was translocated to the centrosomal region only when all components were present, implying that ATP and

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

cytosolic component(s) are required for ZW10 redistribution. In addition, this result confirmed that the ER-associated form of ZW10 is translocated.

ZW10 Redistribution Is Driven by Dynein-Dynactin. Given that ZW10 interacts with dynamitin (Starr et al., 1998), a subunit of dynactin, the most straightforward interpretation of the results described above is that NDGA induces ZW10 redistribution to the centrosome by facilitating its minus-end-directed movement driven by the MT motor dynein-dynactin complex. This idea was supported by the observation that a subunit of the dynactin, $p150^{Glued}$, and dynein IC also accumulated at the centrosomal region upon NDGA treatment (Fig. 2A, +NDGA).

To verify that dynein-dynactin mediates the NDGA-induced movement of ZW10 to the centrosomal region, we first examined the effect of overexpression of dynamitin. Overexpression of dynamitin is known to block dynein-dynactin–mediated processes by disassembling the dynactin complex (Burkhardt et al., 1997). The plasmid for FLAG-dynamitin or, as a control, DsRed, was transfected into HeLa cells, and, 24 h after transfection, the cells were treated with NDGA. As shown in Fig. 2B, NDGA-induced ZW10 redistribution was almost completely inhibited by dynamitin overexpression, but not by DsRed overexpression.

Next, we examined the effect of knockdown of $p150^{Glued}$ on NDGA-induced ZW10 redistribution. HeLa cells were transfected with a short interfering RNA named $p150^{Glued}$ (207) or ZW10 (102), and incubated for 72 h. Immunoblotting revealed that the expression levels of $p150^{Glued}$ and ZW10 were markedly reduced (Fig. 2C). In $p150^{Glued}$-depleted cells, ZW10 did not accumulate at the centrosomal region upon NDGA treatment (Fig. 2C, $p150^{Glued}$ (207)),

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

suggesting that dynactin function might be required for NDGA-induced ZW10 redistribution. It should be noted that depletion of ZW10 did not affect the accumulation of $p150^{Glued}$ at the centrosome (Fig. 2C, ZW10 (102)). This implies that dynactin is required for the redistribution of ZW10, but not vice versa.

NDGA Appears to Stimulate the Association of Dynactin with ZW10. Consistent with the idea that NDGA-induced redistribution of ZW10 is mediated by dynein-dynactin, ZW10 was not accumulated at the centrosomal region when MTs were depolymerized by Noc prior to NDGA treatment. Interestingly, in Noc-, NDGA-treated cells, ZW10 exhibited filamentous structures at the cell periphery. These filaments were positive for the dynactin subunit $p150^{Glued}$ (Fig. 3A), but negative for MTs (data not shown). The formation of these filaments could imply that NDGA stimulates the association between ZW10 and dynactin in a manner that forms an ordered structure. To test whether NDGA influences the association between these proteins, we performed immunoprecipitation using an anti-ZW10 Ab. As shown in Fig. 3A, the amounts of $p150^{Glued}$ and dynein IC co-precipitated with ZW10 were increased in the presence of NDGA, whereas γ-tubulin, which was used as a negative control, was not co-precipitated with ZW10 regardless of whether NDGA was present or not.

To obtain evidence that dynactin is required for the formation of ZW10-positive filaments, the expression of $p150^{Glued}$ was suppressed by RNAi, and then NDGA was added to cells with depolymerized MTs. As shown in Fig. 3B, no obvious ZW10 filaments were observed in $p150^{Glued}$-depleted cells (middle row), whereas $p150^{Glued}$-positive filamentous structures were

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

detected in ZW10-depleted cells (bottom row).

NDGA Induces Centrosomal Accumulation of EB1. Is the action of NDGA specific for ZW10? Alternatively, do other dynactin-interacting proteins also undergo redistribution in the presence of NDGA? To address this question, we investigated the effect of NDGA on the localization of EB1, a MT plus-end tracking protein that is known to interact directly with $p150^{Glued}$ (Berrueta et al., 1999). Without NDGA treatment, EB1 displayed a "comet tail" pattern, representing its predominant association with the growing ends of MTs (Fig. 4, Vehicle). Upon incubation of cells with NDGA, EB1 as well as ZW10 accumulated at the centrosomal region (Fig. 4, +NDGA). As observed for ZW10, NDGA-induced EB1 redistribution was prevented by dynamitin overexpression or depletion of $p150^{Glued}$, and the formation of EB1 filaments observed in Noc-treated cells was dependent on the presence of $p150^{Glued}$ (data not shown).

NDGA Induces Robust Accumulation of Dynein-Dynactin and Its Interacting Proteins at the Spindle Poles. During mitosis, dynein-dynactin and its interacting proteins including ZW10 and EB1 participate in the organization of spindles, spindle checkpoint, and segregation of chromosomes (Chan et al., 2000; Green et al., 2005). We investigated whether the distributions of dynein-dynactin and its interacting proteins in mitotic cells are affected by NDGA, as observed in interphase cells. Incubation of mitotic cells with NDGA resulted in robust accumulation of these proteins at the spindle poles (Fig. 5A +NDGA). Remarkably, almost all cells treated with NDGA displayed a metaphase-like pattern. Moreover, a significant fraction of NDGA-treated cells showed aberrant chromosome distribution. Chromosomes were localized, in addition to at the

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

metaphase plate, at or near spindle poles (Fig 5B, +NDGA) or outside of the spindles (data not shown).

Effect of NDGA on the Distribution of Tf receptor (TfR). Dynein-dynactin is present on endosomes (Habermann et al., 2000) and endocytosis of Tf is blocked by disruption of dynein-dynactin function (Burkhardt et al., 1997). As NDGA inhibits the endocytic pathway (Ramoner et al., 1998), we examined whether the localization of TfR is affected by NDGA. In control cells, TfR was distributed throughout the cytoplasm with some concentration in the perinuclear region, which may represent recycling endosomes (Fig. 6A, Vehicle). Upon incubation with NDGA, TfR accumulated in the perinuclear region with a marked loss of peripheral localization (Fig. 6A, +NDGA). Consistent with a previous study (Ramoner et al., 1998), uptake of Tf-FITC was blocked in the presence of NDGA (Fig. 6B, +NDGA). Furthermore, recycling of Tf-FITC, i.e., release of incorporated Tf-FITC into the medium, was also blocked by NDGA, and the Tf-FITC remained colocalized with TfR in the perinuclear region (Fig. 6C, +NDGA). This phenotype is in marked contrast to that in cells overexpressing dynamitin. In dynamitin-overexpressing cells, uptake of Tf occurs but its movement to the cell center is blocked (Burkhardt et al., 1997).

Uptake and recycling of Tf are arrested during mitosis (Sager et al., 1984). We were interested in whether TfR distribution in mitotic cells is affected by NDGA. In mitotic cells, TfR was found to be distributed diffusely throughout the cell with some concentration at the spindle poles (Fig. 6D, Vehicle). In marked contrast to the case of interphase cells, addition of NDGA did

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

not significantly affect the localization of TfR (Fig. 6D, +NDGA). This may imply that the connection between TfR-containing endosomes and dynein-dynactin is regulated in a cell cycle-dependent manner. Indeed, previous work demonstrated that dynein-dynactin detaches from membranes in metaphase *Xenopus* egg extracts (Niclas et al., 1996).

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

In the present study, we demonstrated that NDGA induces the accumulation of dynein-dynactin at the centrosome in interphase cells and at the spindle poles in mitotic cells. Concomitant with the movement of dynein-dynactin, dynactin-interacting proteins, such as ZW10 and EB1, were also transported, depending on cell cycle, to the centrosomal region or the spindle poles. Upon NDGA treatment, TfR was also redistributed to the centrosomal region in interphase cells. Loss of dynactin function caused by disassembly of dynactin by dynamitin overexpression or depletion of the dynactin subunit $p150^{Glued}$ abrogated the NDGA-induced movement of dynactin-interacting components to the centrosomal region, whereas depletion of ZW10 had no effect on NDGA-induced redistribution of dynein-dynactin. These results suggest that NDGA can affect dynein-dynactin function and facilitate processes mediated by this motor.

The Mechanism for NDGA Action on Dynein-dynactin and Its Interacting Proteins. How can NDGA induce the movement of dynein-dynactin and its interacting proteins to the centrosome? The phenotype of cells with depolymerized MTs provides a clue to understanding the mechanism for this NDGA action. When MTs were depolymerized, NDGA promoted the formation of filaments comprising dynein-dynactin, ZW10 and EB1. The filament formation in the absence of intact MTs most likely reflects the enhanced association between dynein-dynactin and its interacting proteins. Perhaps, the NDGA-induced association of these proteins is highly ordered because NDGA treatment of Noc-treated cells did not induce aggregation, which is a hallmark of nonspecific protein-protein interactions. The result of immunoprecipitation analysis

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

supported the view that NDGA strengthens the association between dynein-dynactin and ZW10. Given that dynactin functions not only as a cargo adaptor but also as a factor conferring dynein processivity (Culver-Hanlon et al., 2006), it is tempting to speculate that the enhanced association between dynein-dynactin and its interacting proteins allows a long-range movement of these proteins toward the minus-end of MTs, leading to their accumulation at the centrosomal region.

Mechanism of Golgi Disassembly and Endocytosis Inhibition by NDGA. Our results suggest that Golgi disassembly and blockage of endocytosis induced by NDGA are due to the excessive stimulation of dynein-dynactin-mediated processes. This view can explain the finding by Fujiwara et al. (1998b) that, upon NDGA treatment, a marker for the ER-Golgi intermediate compartment, ERGIC-53, rapidly moves to the perinuclear, centrosomal region prior to Golgi enzymes to be redistributed to the ER. This movement of ERGIC-53 is likely coupled to the movement of dynein-dynactin to the centrosomal region. The ER-Golgi intermediate compartment coalescences with the Golgi to form aggregated membrane structures, which may fuse directly with proximal ER membranes (Fujiwara et al., 2003). In the case of endocytosis, TfR-containing endosomes, in association with dynein-dynactin, accumulate at the centrosomal region, leading to a deficiency of TfR at the plasma membrane.

Based on the observation that activation of trimeric GTP-binding proteins prevents NDGA-induced Golgi disassembly, we previously suggested that this reagent affects the function of trimeric GTP-binding proteins (Yamaguchi et al., 1997). However, as dynein-dynactin and its interacting proteins accumulated at the centrosomal region upon NDGA treatment irrespective of

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

the presence or absence of aluminum fluoride (data not shown), the previous interpretation should be modified. Perhaps, activation of GTP-binding proteins stabilizes Golgi membranes by extensively recruiting peripheral protein complexes to the membranes so that lateral movement of Golgi membrane proteins is constrained (Cole et al., 1996). The stabilized Golgi apparatus can maintain its structure even when large amounts of dynein-dynactin and its interacting proteins accumulate at the centrosomal region.

NDGA as a Drug to Regulate MT Stability and MT-related Processes. NDGA, unlike taxol, does not promote MT polymerization, although it stabilizes MTs (Nakamura et al., 2003). Based on the result of an indirect measurement, Nakamura et al. (2003) suggested that NDGA prevents MT depolymerization by directly binding to tubulin. However, no tubulin was found in the filaments formed upon NDGA treatment of Noc-treated cells, suggesting that the target for NDGA is, at least, not limited to tubulin. As plus-end tracking proteins, such as EB1 and dynactin, play a role in regulating MT dynamics (Carvalho et al., 2003), it is possible that the NDGA, in addition to its direct binding to tubulin, indirectly stabilizes MTs by regulating the function of plus-end tracking proteins.

The action of NDGA on MTs and/or their associated proteins may provide insight into the action of NDGA, not as a lipoxygenase inhibitor or an antioxidant. Lee et al. (2003) reported that NDGA, but not other antioxidants, inhibits transforming growth factor-β activity by blocking the phosphorylation and nuclear translocation of Smad2. This effect of NDGA can be explained by the fact that Smad2 binds to MTs (Dong et al., 2000). NDGA may block transforming growth

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

factor-β activity by stabilizing MTs or affecting MT dynamics. Indeed, depolymerization of MTs by Noc was found to induce the phosphorylation and nuclear translocation of Smad2 (Dong et al., 2000). As many transcription factors and protein kinases interact with MTs (Gundersen and Cook, 1999), NDGA may influence transcription and signal transduction by affecting the stability of MTs and/or dynein-dynactin function.

MT-stabilizing reagents such as taxol have been successfully used in the treatment of solid tumors (Bergstralh and Ting, 2006). The suppression of MT dynamics disrupts the mitotic spindle, halting the cell cycle at the metaphase-anaphase and eventually leading to apoptosis (Yvon et al., 1999). In the presence of NDGA, the mitotic spindle appeared not to be substantially disrupted. The different effects of taxol and NDGA on the mitotic spindle are consistent with the observations that NDGA does not affect the radiation of MTs originating from the centrosome in interphase cells, whereas taxol perturbs MT array (Nakamura et al., 2003). The misalignment of chromosomes induced by NDGA might be due to premature removal of spindle checkpoint proteins, such as ZW10, from kinetochores. Although the mechanisms of stabilization of MTs by taxol and NDGA are probably different, the stabilization of MT by NDGA also seems to halt the cell cycle at the metaphase-anaphase. This may explain why NDGA causes apoptosis in several different tumor xenografts (Avis et al., 1996; Seufferlein et al., 2002).

In summary, we disclosed a novel action of NDGA, i.e. stimulation of processes mediated by the MT motor dynein-dynactin complex. The anti-cancer and other drug activities of NDGA should be investigated in the light of MT-related processes.

References

Avis IM, Jett M, Boyle T, Vos MD, Moody T, Treston AM, Martinez A, and Mulshine JL (1996) Growth control of lung cancer by interruption of 5-lipoxygenase-mediated growth factor signaling. *J Clin Invest* **197:**806-813.

Bergstralh DT, and Ting JPY (2006) Microtubule stabilizing agents: Their molecular signaling consequences and the potential for enhancement by drug combination. *Cancer Treat Rev* **32:**166-179.

Berrueta L, Tirnauer JS, Schuyler SC, Pellman D, and Bierer BE (1999) The APC-associated protein EB1 associates with components of the dynactin complex and cytoplasmic dynein intermediate chain. *Curr Biol* **9:** 425–428.

Burkhardt JK, Echeverri CJ, Nilsson T, and Vallee RB (1997) Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J Cell Biol* **139:**469-484.

Carvalho P, Tirnauer JS, and Pellman D (2003). Surfing on microtubule ends. *Trends Cell Biol* **13:**229-237.

Chan GKT, Jablonski SA, Starr DA, Goldberg ML, and Yen TJ (2000) Human Zw10 and ROD are mitotic checkpoint proteins that bind to kinetochores. *Nature Cell Biol* **2:**944-947.

Cole NB, Smith CL, Sciaky N, Terasaki M, Edidin M, and Lippincott-Schwartz J (1996) Diffusional mobility of Golgi proteins in membranes of living cells. *Science* **273:**797-801.

Culver-Hanlon TL, Lex SA, Stephens AD, Quintyne NJ, and King SJ (2006) A

microtubule-binding domain in dynactin increases dynein processivity by skating along microtubules. *Nat Cell Biol* **8:**264-270.

Domin J, Higgins T, and Rozengurt E (1994) Preferential inhibition of platelet-derived growth factor-stimulated DNA synthesis and protein tyrosine phosphorylation by nordihydroguaiaretic acid. *J Biol Chem* **269:**8260-8267.

Dong C, Li Z, Alvarez R Jr, Feng XH, and Goldschmidt-Clemont PJ (2000) Micorotubule binding to Smads may regulate TGF-β activity. *Mol Cell* **5:**27-34.

Drecktrah D, de Figueiredo P, Mason RM, and Brown WJ (1998) Retrograde trafficking of both Golgi complex and TGN markers to the ER induced by nordihydroguaiaretic acid and cyclofenil diphenol. *J Cell Sci* **111:**951-965.

Fujiwara T, Takami N, Misumi Y, and Ikehara Y (1998a) Nordihydroguaiaretic acid blocks protein transport in the secretory pathway causing redistribution of Golgi proteins into the endoplasmic reticulum. *J Biol Chem* **273:**3068-3075.

Fujiwara T, Misumi Y, and Ikehara Y (1998b) Dynamic recycling of ERGIC53 between the endoplasmic reticulum and the Golgi complex is disrupted by nordihydroguaiaretic acid. *Biochem Biophys Res Commun* **253:**869-876.

Fujiwara T, Misumi Y, and Ikehara Y (2003) Direct interaction of the Golgi membrane with the endoplasmic reticulum membrane caused by nordihydroguaiaretic acid. *Biochem Biophys Res Commun* **301:**927-933.

Gnabre JN, Brady JN, Clanton DJ, Ito Y, Dittmer J, Bates RB, and Huang RC (1995) Inhibition

of human immunodeficiency virus type 1 transcription and replication by DNA sequence-selective plant lignans. *Proc Natl Acad Sci USA* **92:**11239-11243.

Goetzl EJ (1980) A role for endogenous mono-hydroxy-eicosatetraenoic acids (HETEs) in the regulation of human neutrophil migration. *Immunology* **40:**709-719.

Green RA, Wollman R, and Kaplan KB (2005) APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. *Mol Biol Cell* **16:**4609-4622.

Gundersen GG, and Cook TA (1999) Microtubules and signal transduction. *Curr Opin Cell Biol* **11:** 81-94.

Habermann A, Schoroer TA, Griffiths G, and Burkhardt JK (2000) Immunolocalization of cytoplasmic dynein and dynactin subunits in cultured macrophages: enrichment on early endocytoic organelles. *J Cell Sci* **114:** 229-240.

Hirose H, Arasaki K, Dohmae N, Takio K, Hatsuzawa K, Nagahama M, Tani K, Yamamoto A, Tohyama M, and Tagaya M (2004) Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi. *EMBO J* **23:**1267-1278.

Huang JK, Chen WC, Huang CJ, Hsu SS, Chen JS, Cheng HH, Chang HT, Jiann BP, and Jan CR (2004) Nordihydroguaiaretic acid-induced Ca^{2+} handling and cytotoxicity in human prostate cancer cells. *Life Sci* **5:**2341-2351.

Ito H, Ueda H, Iwamoto I, Inaguma Y, Takizawa T, Asano T, and Kato K (2005) Nordihydroguaiaretic acid (NDGA) blocks the differentiation of C2C12 myoblast cells. *J Cell Physiol* **202:**874-879.

Korn SJ, and Horn R (1990) Nordihydroguaiaretic acid inhibits voltage-activated Ca^{2+} currents independently of lipoxygenase inhibition. *Mol Pharmacol* **38:**524-530.

Lee CH, Jang YS, Her SJ, Moon YM, Baek SJ, and Eling T (2003) Nordihydroguaiaretic acid, an antioxidant, inhibits transforming growth factor-beta activity through the inhibition of Smad signaling pathway. *Exp Cell Res* **289:**335-341.

McDonald RW, Bunjobpon W, Liu T, Fessler S, Pardo OE, Freer IK, Glaser M, Seckl MJ, and Robins DJ (2001) Synthesis and anticancer activity of nordihydroguaiaretic acid (NDGA) and analogues. *Anticancer Drug Des* **16:**261-270.

Moss MA, Varvel NH, Nichols MR, Reed DK, and Rosenberry TL (2004) Nordihydroguaiaretic acid does not disaggregate β-amyloid(1-40) protofibrils but does inhibit growth arising from direct protofibril association. *Mol Pharmcol* **66:**592-600

Nakamura M, Nakazawa J, Usui T, Osada H, Kono Y, and Takatsuki A (2003) Nordihydroguaiaretic acid, of a new family of microtubule-stabilizing agents, shows effects differentiated from paclitaxel. *Biosci Biotechnol Biochem* **67:** 151-157.

Niclas J, Allan VJ, and Vale RD (1996) Cell cycle regulation of dynein association with membranes modulates microtubule-based organelle transport. *J Cell Biol* **133:**585-593.

Ramasamy S, Drummond GR, Ahn J, Storek M, Pohl J, Parthasarathy S, and Harrison DG (1999) Modulation of expression of endothelial nitric oxide synthase by nordihydroguaiaretic acid, a phenolic antioxidant in cultured endothelial cells. *Mol Pharmacol* **56:**116-123.

Ramoner R, Rieser C, Bartsch G, and Thurnher M (1998) Nordihydroguaiaretic acid blocks secretory and endocytic pathways in human dendritic cells. *J Leukoc Biol* **164:**747-752.

Sager PR, Brown, PA, and Berlin RD (1984) Analysis of transferrin recycling in mitotic and interphase HeLa cells by quantitative fluorescence microscopy. *Cell* **39:**275-282.

Starr DA, Williams BC, Hays TS, and Goldberg ML (1998) ZW10 helps recruit dynactin and dynein to the kinetochore. *J Cell Biol* **142:**763-774.

Seufferlein T, Seckl MJ, Schwarz E, Beil M, v Wichert G, Baust H, Luhrs H, Schmid RM, and Adler G (2002) Mechanisms of nordihydroguaiaretic acid-induced growth inhibition and apoptosis in human cancer cells. *Br J Cancer* **86:**1188-1196.

Tagaya M, Henomatsu N, Yoshimori T, Yamamoto A, Tashiro Y, and Fukui T (1993) Correlation between phospholipase A_2 activity and intra-Golgi protein transport reconstituted in a cell-free system. *FEBS Lett* **324:**201-204.

Tagaya M, Henomatsu N, Yoshimori T, Yamamoto A, Tashiro Y, and Mizushima S (1996) Inhibition of vesicle-mediated protein transport by nordihydroguaiaretic acid. *J Biochem* **119:**863-869.

West M, Mhatre M, Ceballos A, Floyd RA, Grammas P, Gabbita SP, Hamdheydari L, Mai T, Mou S, Pye QN, Stewart C, West S, Williamson KS, Zemlan F, and Hensley K (2004) The arachidonic acid 5-lipoxygenase inhibitor nordihydroguaiaretic acid inhibits tumor necrosis factor alpha activation of microglia and extends survival of G93A-SOD1 transgenic mice. *J Neurochem* **91:**133-143.

Yamaguchi T, Yamamoto A, Furuno A, Hatsuzawa K, Tani K, Himeno M, and Tagaya M (1997) Possible involvement of heterotrimeric G proteins in the organization of the Golgi apparatus. *J Biol Chem* **272:**25260-25266.

Yoshimori T, Shimonishi Y, and Uchida T (1988). Binding properties of monoclonal antibody to the cytoplasmic domain of transferrin receptor. *Cell Struct Funct* **113:** 311-324.

Youngren JF, Gable K, Penaranda C, Maddux BA, Zavodovskaya M, Lobo M, Campbell M, Kerner J, and Goldfine ID (2005) Nordihydroguaiaretic acid (NDGA) inhibits the IGF-1 and c-erbB2/HER2/neu receptors and suppresses growth in breast cancer cells. *Breast Cancer ResTreat* **94:**37-46.

Yvon AM, Wadsworth P, and Jordan MA (1999) Taxol suppresses dynamics of individual microtubules in living human tumor cells. *Mol Biol Cell* **10:**947-959.

(Footnotes)

This work was supported in part by Grants-in-Aid for Scientific Research (#16370089, #16044242, #16048229, and #16657309) from the Ministry of Education, Science, Sports and Culture of Japan. K. A. is a research fellow of the Japan Society for the Promotion of Science.

Reprint requests should be sent to Dr. Mitsuo Tagaya, School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan. Tel: +81-42-676-5419; Fax: +81-42-676-5468; E-mail: tagaya@ls.toyaku.ac.jp.

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

(Legends for Fgiures)

Fig. 1. Characterization of NDGA-induced ZW10 redistribution. A, HeLa cells were treated for 1 h with 0.1% DMSO (Vehicle) or 30 μM NDGA (+NDGA) and then double stained with Abs against ZW10 and GM130 (top and middle rows) or γ-tubulin (bottom row). N denotes the position of the nucleus. Bar, 5 μm. B, HeLa cells were treated with 0.1% DMSO (Vehicle) for 1 h, 20 μM α-tocopherol for 5 h, 1 mM ascorbic acid for 5 h, 1 mM *N*-acetyl cysteine (NAC) for 5 h, 20 μM 5, 8, 11, 14-eicosatetraynoic acid (ETYA) for 3 h, or 10 μg/ml taxol for 2 h. Fetal calf serum was omitted during incubation with these reagents. The cells were stained with an Ab against ZW10. Bar 5 μm. C, Requirement of energy and cytosolic factor(s) for ZW10 redistribution. Digitonin-permeabilized HeLa cells expressing ZW10-GFP were incubated at 32°C for 60 min without or with 30 μM NDGA in the presence of cytosol (Cy) and/or an ATP-regenerating system (ATP). Arrows indicate the position of centrosomal ZW10-GFP. Bar, 5 μm. The quantitative results are shown on the right. Error bars represent the standard error of the mean for three experiments.

Fig. 2. Dynactin function is required for NDGA-induced ZW10 redistribution. A, HeLa cells were treated with 0.1% DMSO (Vehicle) or 30 μM NDGA (+NDGA) for 1 h and then double stained with Abs against ZW10 and $p150^{Glued}$ or dynein IC. Bar, 5 μm. B, HeLa cells were transfected with the plasmid for FLAG-dynamitin or DsRed. After 24 h, the cells were treated with 30 μM NDGA for 1 h and then double stained with Abs against ZW10 and FLAG (upper

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

row) or only stained for ZW10 (lower row). Although methanol treatment failed to fix most of the expressed DsRed, DsRed-expressing cells were recognizable because of the presence of DsRed remnants. Bar, 5 μm. The quantitative results are shown on the right. Error bars represent the standard error of the mean for three experiments. C, HeLa cells were transfected without (Mock) or with $p150^{Glued}$ (207) or ZW10 (102). At 72 h after transfection, the cells were solubilized in phosphate-buffered saline with 0.5% SDS and analyzed by immunoblotting. Alternatively, the transfected cells were incubated for 1 h with 0.1% DMSO (Vehicle) or 30 μM NDGA (+NDGA) and then double stained with Abs against $p150^{Glued}$ and ZW10. Bar, 5 μm.

Fig. 3. Dynactin is required for ZW10 filament formation induced by NDGA. A, HeLa cells were pretreated with 10 μg/ml Noc for 1 h to depolymerize MTs. 30 μM NDGA was added, and then the cells were incubated for 1 h and double stained with Abs against ZW10 and $p150^{Glued}$. The boxed area is shown on an expanded scale. Bar, 5 μm. For an immunoprecipitation experiment, 293T cells were incubated for 1 h with 0.1% DMSO (Vehicle) or 30 μM NDGA (+NDGA), or pretreated with 10 μg/ml Noc for 1 h followed by incubation with 10 μg/ml Noc plus 30 μM NDGA for 1 h (+Noc/NDGA). Cell lysates were prepared, immunoprecipitated with an anti-ZW10 Ab and analyzed by immunoblotting with the indicated Abs. Asterisks represent immunoglobulin heavy chain. B, $p150^{Glued}$ or ZW10 was knocked down as described in the legend to Fig. 2C. The cells were incubated with 10 μg/ml Noc for 90 min, and then with 10 μg/ml Noc plus 30 μM NDGA for 1 h. The cells were stained with Abs against $p150^{Glued}$ and

ZW10. Arrowheads indicate the position of typical filamentous structures. Bar, 5 μm.

Fig. 4. Distribution of EB1 in NDGA-treated cells. HeLa cells were treated for 1 h with 0.1% DMSO (Vehicle) or 30 μM NDGA (+NDGA) and then double stained with Abs against EB1 and ZW10. Bar, 5 μm.

Fig. 5. Effect of NDGA on the distribution of dynein-dynactin and its interacting proteins in mitotic cells. HeLa cells were synchronized by double thymidine block. At 6 h after washing out thymidine, the cells were incubated for 1 h with 0.1% DMSO (Vehicle) or 30 μM NDGA (+NDGA) and then double stained with the indicated Abs (A) or with ZW10 and propidium iodide (PI) (B). Bar, 5 μm. The percentage of cells with abnormal chromosome alignment was scored. Error bars represent the standard error of the mean for four experiments.

Fig. 6. Redistribution of TfR by NDGA. A, HeLa cells were incubated for 1 h with 0.1% DMSO (Vehicle) or 30 μM NDGA (+NDGA), fixed with 4% paraformaldehyde at room temperature for 20 min and double stained with Abs against TfR and a Golgi marker, syntaxin 5 (Syn 5). Bar, 5 μm. B, HeLa cells were preincubated in the absence of fetal calf serum for 1 h to remove endogenous Tf, and then 0.1% DMSO (Vehicle) or 30 μM NDGA (+NDGA) was added. After a 1-h incubation, Tf-FITC was added to a final concentration of 25 μg/ml, and the incubation was continued for another 1 h. The cells were stained with an Ab against TfR. C, HeLa cells were

Molecular Pharmacology Fast Forward. Published on November 14, 2006 as DOI: 10.1124/mol.106.029611
This article has not been copyedited and formatted. The final version may differ from this version.

incubated with 25 μg/ml Tf-FITC for 1 h to allow the uptake of ligand. The cells were washed and incubated with 0.1% DMSO (Vehicle) or 30 μM NDGA (+NDGA). After a 1-h incubation, the cells fixed and stained with an Ab against TfR. D, HeLa cells were synchronized by double thymidine block. At 6 h after washing out thymidine, the cells were treated for 1 h with 0.1% DMSO (Vehicle) or 30 μM NDGA (+NDGA) and then double stained with Abs against TfR and α-tubulin. Bar, 5 μm.

Figure 1

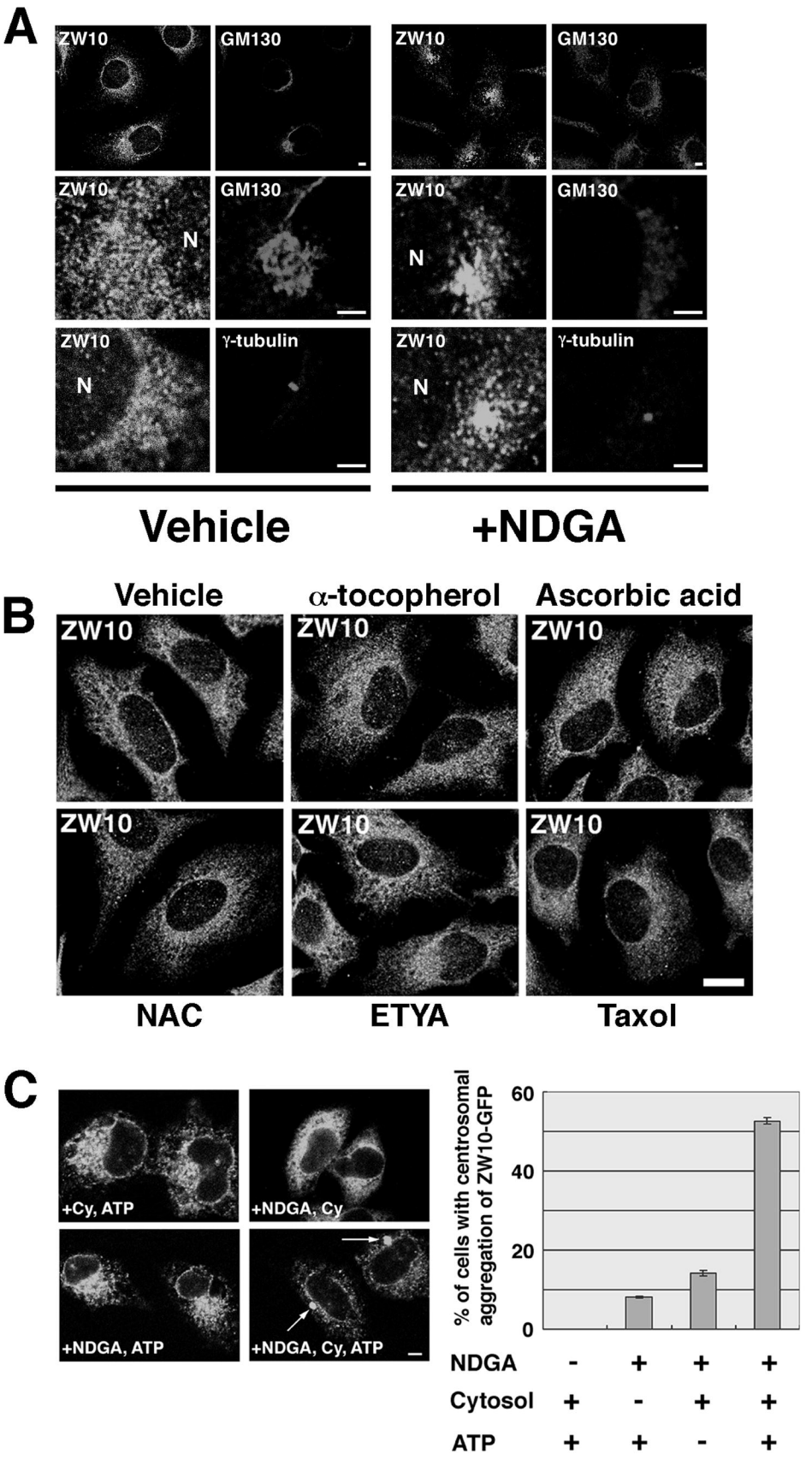

A
ZW10
GM130
ZW10
GM130
ZW10
N
GM130
ZW10
GM130
ZW10
N
GM130
ZW10
N
γ-tubulin
ZW10
N
γ-tubulin
Vehicle
+NDGA
B
Vehicle
α-tocopherol
Ascorbic acid
ZW10
ZW10
ZW10
ZW10
ZW10
ZW10
NAC
ETYA
Taxol
C
+Cy, ATP
+NDGA, Cy
+NDGA, ATP
+NDGA, Cy, ATP
% of cells with centrosomal aggregation of ZW10-GFP
60
40
20
0
NDGA - + + +
Cytosol + - + +
ATP + + - +

Figure 2

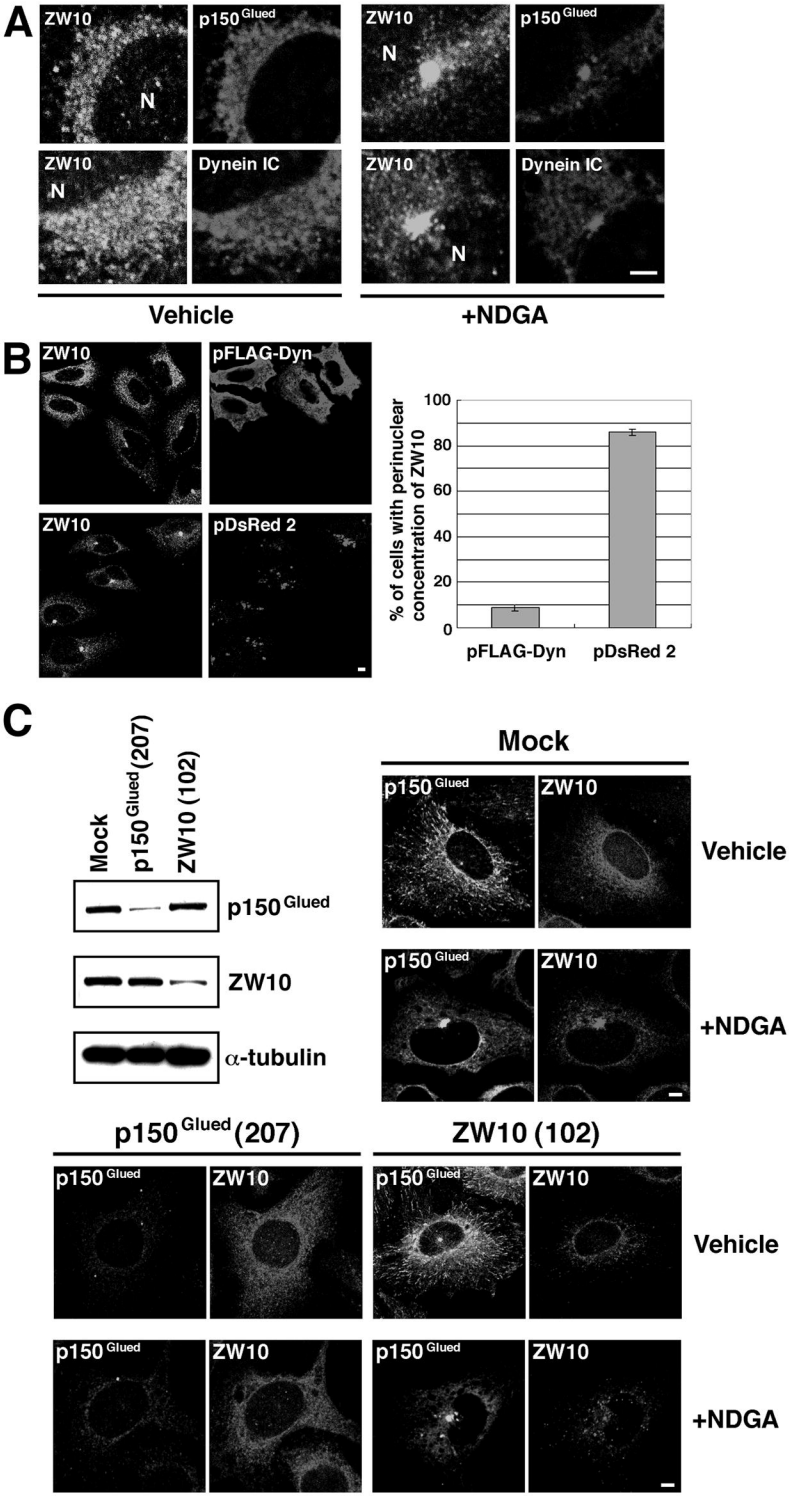
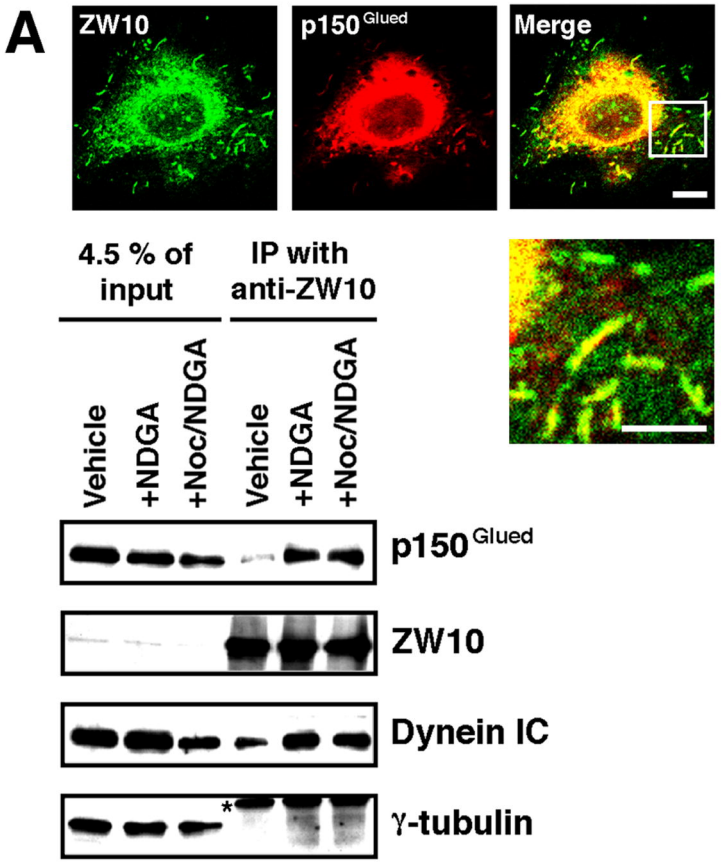

A
ZW10
p150 Glued
N
ZW10
Dynein IC
N
Vehicle
+NDGA
B
ZW10
pFLAG-Dyn
ZW10
pDsRed 2
% of cells with perinuclear concentration of ZW10
100
80
60
40
20
0
pFLAG-Dyn
pDsRed 2
C
Mock
p150 Glued (207)
ZW10 (102)
p150 Glued
ZW10
α-tubulin
Mock
Vehicle
+NDGA
p150 Glued (207)
ZW10 (102)

Figure 3


A
ZW10
p150 Glued
Merge
4.5 % of input
IP with anti-ZW10
Vehicle
+NDGA
+Noc/NDGA
Vehicle
+NDGA
+Noc/NDGA
p150 Glued
ZW10
Dynein IC
γ-tubulin
*

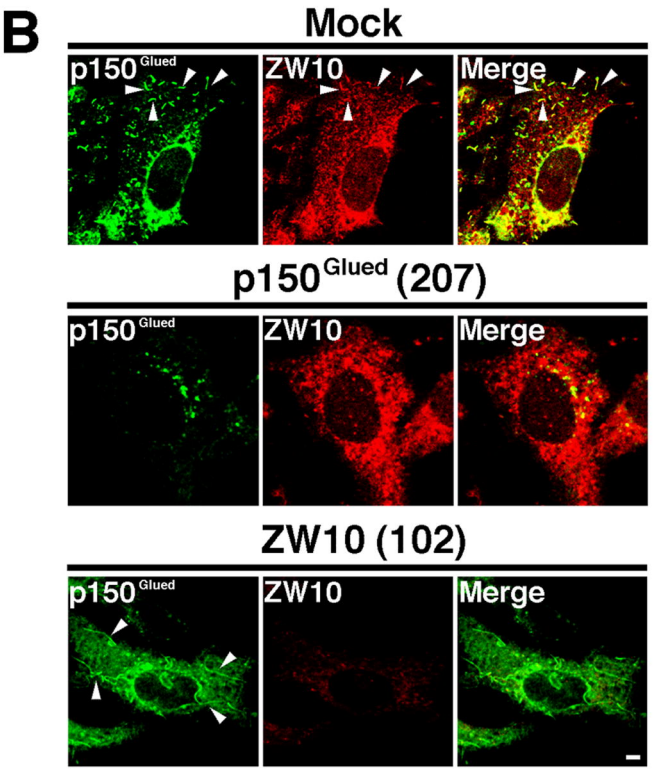

B
Mock
p150 Glued
ZW10
Merge
p150 Glued (207)
p150 Glued
ZW10
Merge
ZW10 (102)
p150 Glued
ZW10
Merge

Figure 4

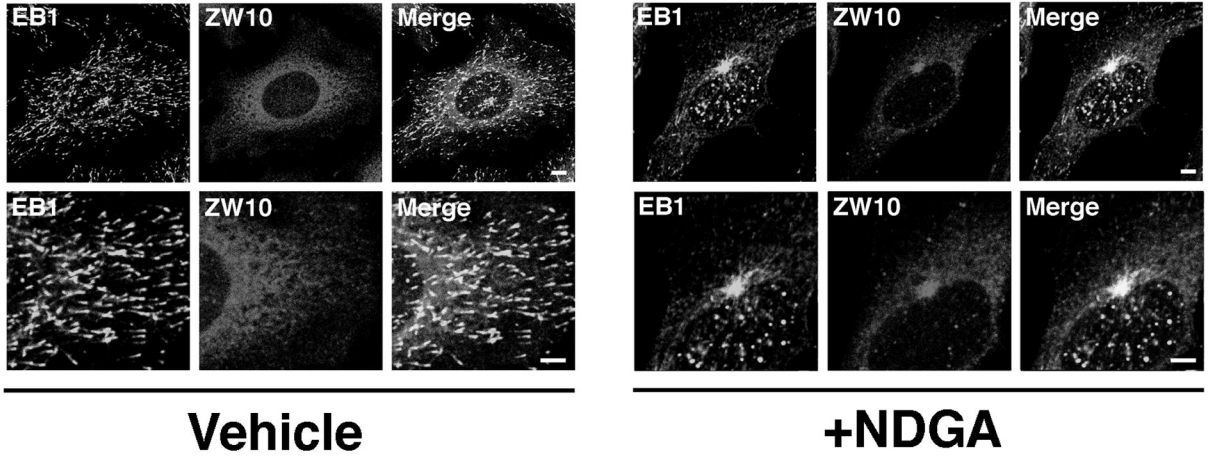
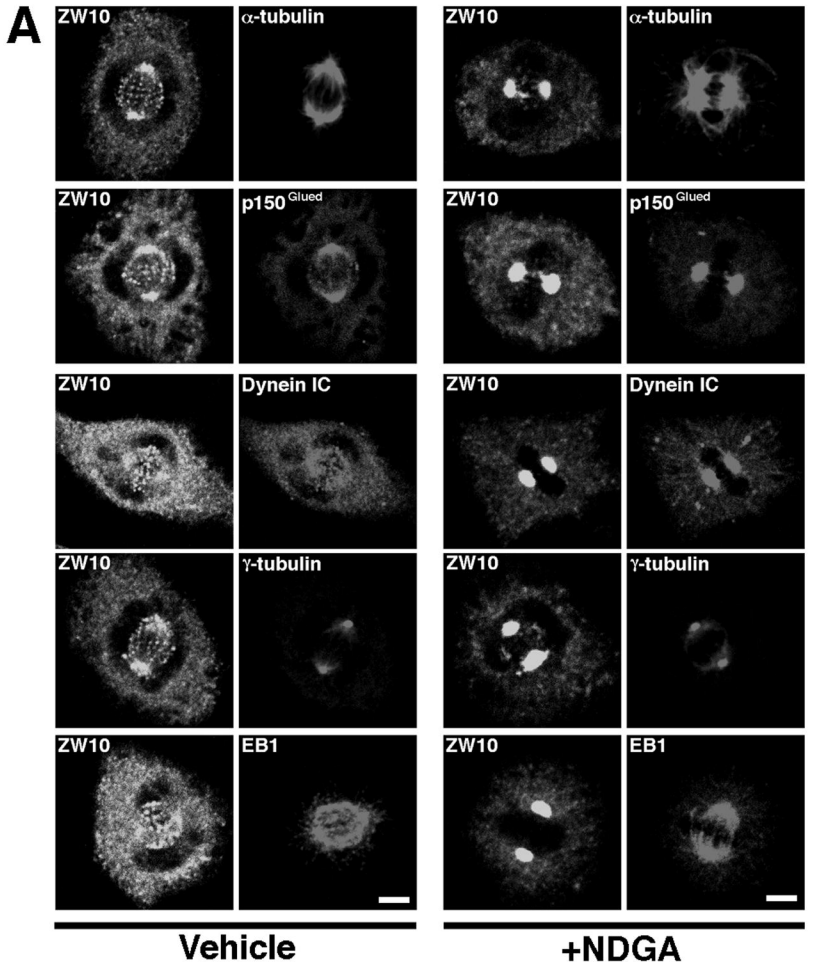

EB1
ZW10
Merge
EB1
ZW10
Merge
EB1
ZW10
Merge
EB1
ZW10
Merge
Vehicle
+NDGA

Figure 5


A
ZW10
α-tubulin
ZW10
α-tubulin
ZW10
p150 Glued
ZW10
p150 Glued
ZW10
Dynein IC
ZW10
Dynein IC
ZW10
γ-tubulin
ZW10
γ-tubulin
ZW10
EB1
ZW10
EB1
Vehicle
+NDGA

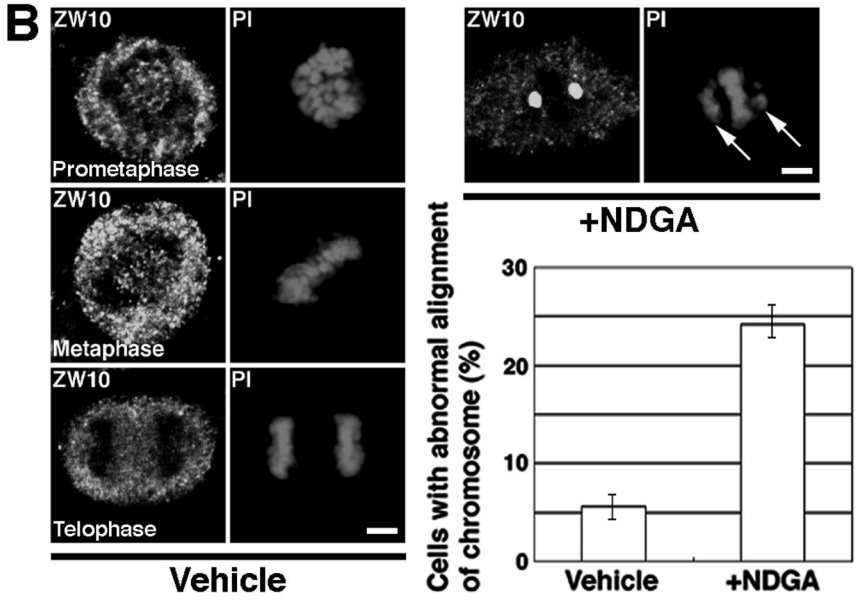
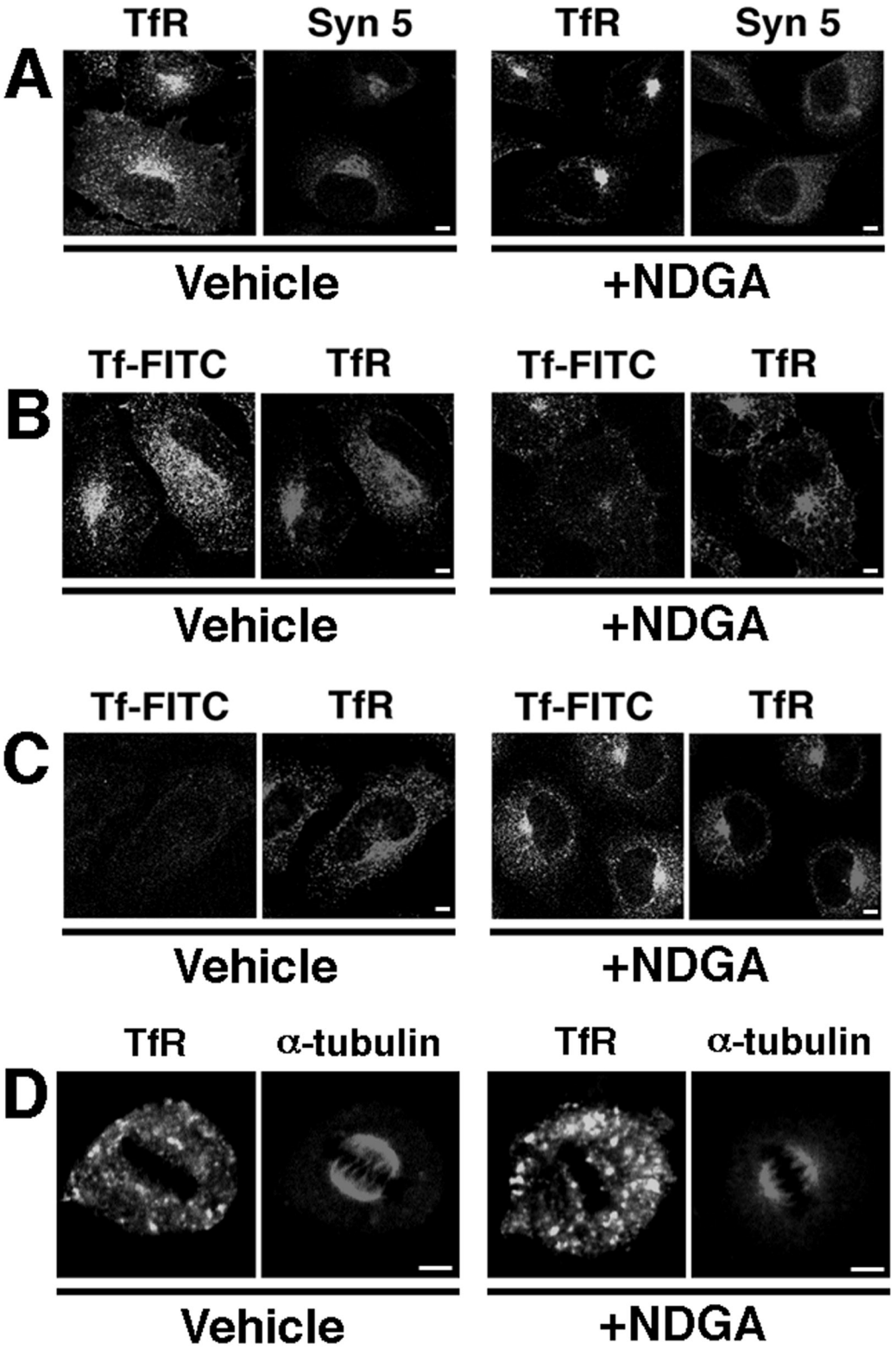

B
ZW10
PI
Prometaphase
ZW10
PI
Metaphase
ZW10
PI
Telophase
Vehicle
ZW10
PI
+NDGA
Cells with abnormal alignment of chromosome (%)
30
20
10
0
Vehicle
+NDGA

Figure 6


A
TfR
Syn 5
TfR
Syn 5
Vehicle
+NDGA
B
Tf-FITC
TfR
Tf-FITC
TfR
Vehicle
+NDGA
C
Tf-FITC
TfR
Tf-FITC
TfR
Vehicle
+NDGA
D
TfR
α-tubulin
TfR
α-tubulin
Vehicle
+NDGA