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Disruption of Microtubules in Living Cells by Tyrphostin AG-1714

Tova Volberg,[1] Alexander D. Bershadsky,[1] Michael Elbaum,[2] Aviv Gazit,[3] Alexander Levitzki,[4] and Benjamin Geiger[1]*

[1]*Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel*
[2]*Department of Materials and Interfaces, The Weizmann Institute of Science, Rehovot, Israel*
[3]*Department of Organic Chemistry, The Alexander Silberman Institute for Life Sciences, The Hebrew University, Jerusalem, Israel*
[4]*Institute of Chemistry and Biological Chemistry, The Alexander Silberman Institute for Life Sciences, The Hebrew University, Jerusalem, Israel*

Tyrphostin AG-1714 and several related molecules with the general structure of nitro-benzene malononitrile (BMN) disrupt microtubules in a large variety of cultured cells. This process can be inhibited by the stabilization of microtubules with taxol or by pretreatment of the cells with pervanadate, which inhibits tyrosine phosphatases and increases the overall levels of phosphotyrosine in cells. Unlike other microtubule-disrupting drugs such as nocodazole or colchicine, tyrphostin AG-1714 does not interfere with microtubule polymerization or stability in vitro, suggesting that the effect of this tyrphostin on microtubules is indirect. These results imply an involvement of protein tyrosine phosphorylation in the regulation of overall microtubule dynamics. Tyrphostins of AG-1714 type could thus be powerful tools for the identification of such microtubule regulatory pathways. Cell Motil. Cytoskeleton 45:223–234, 2000.

Key words: microtubule dynamics; tyrphostins; tyrosine phosphorylation; taxol; pervanadate

INTRODUCTION

The microtubular system plays a central role in a wide variety of dynamic processes in living cells including transport of vesicles and organelles, chromosome movement in mitosis, and polarized formation of pseudopodial extensions, as well as in maintenance of the overall cell shape and polarity [Hyams and Lloyd, 1994]. These complex functions of microtubules are controlled by two general mechanisms. The first is the active translocation of various "cargoes" such as vesicles or chromosomes along microtubules, driven by specific motor molecules of the kinesin and dynein families [Hirokawa, 1998]. The second involves dynamic changes in the length of microtubules and modulation of their assembly and disassembly [Gelfand and Bershadsky, 1991; Desai and Mitchison, 1997; Joshi, 1998; Andersen, 1999]. These mechanisms operate at several levels. First, the association of the tubulin assembly with GTP-hydrolysis makes microtubule assembly a dissipative rather than equilibrium process, and drives a so-called "dynamic instability" behavior. This means that microtubule length can oscillate due to alternating periods of elongation and rapid ("catastrophic") shortening. The dynamic instability behavior is an intrinsic characteristic of tubulin itself

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Correspondence concerning tyrphostins: Alexander Levitzki
E-mail: levitzki@vms.huji.ac.il

*Correspondence to: Benjamin Geiger, Dept. of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel.
E-mail: ligeiger@wicc.weizmann.ac.il

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and can be demonstrated in vitro [Horio and Hotani, 1986; Walker et al., 1988]. In the majority of mitotic cells, the "minus" ends of microtubules are stabilized by interactions with the centrosome [Kellogg et al., 1994], and microtubule turnover is mediated by the dynamic instability at the peripheral "plus" ends. If a microtubule is detached from the centrosome, an alternative dynamic regime is possible: the microtubule can depolymerize at the "minus" and grow at the "plus" end. This type of dynamic behavior called "treadmilling" was observed in microtubules of centrosome-free cell fragments in interphase [Rodionov and Borisy, 1997].

Dynamics of microtubules in vivo is regulated by a variety of microtubule-associated cellular components. Among these are γ-tubulin-containing complexes that nucleate microtubules polymerization [Kellogg et al., 1994; Hyman and Karsenti, 1998], and proteins that stabilize microtubules by binding to the microtubule wall (classical MAPs) [Mandelkow and Mandelkow, 1995] or by capping its ends [Denarier et al., 1998]. Other proteins were shown to have the opposite effect, inducing microtubule depolymerization or disintegration into short fragments. In particular, a kinesin-like protein XKCM1 promotes depolymerization of the microtubules from the "plus" end [Walczak et al., 1996], a protein known as katanin severs microtubules [McNally and Vale, 1993; McNally et al., 1996], and the protein stathmin, or Op18, destabilizes microtubules by sequestering tubulin subunits [Curmi et al., 1997] and increasing their catastrophe frequency [Belmont and Mitchison, 1996]. Some proteins, such as XMAP 215, promote both elongation and shortening of microtubules, resulting in an increase in polymer turnover [Vasquez et al., 1994].

In addition, interactions of microtubules with other cellular components, including other cytoskeletal structures, can also affect their dynamics. For example, actomyosin-based retrograde flow can break microtubules, forming a population of non-centrosomal microtubules whose turnover is regulated at their "minus" ends [Waterman-Storer and Salmon, 1997, 1999]. Association of microtubules with early focal adhesions, on the other hand, can protect them from depolymerization [Kaverina et al., 1998].

Complex and well-coordinated regulation of microtubule dynamics is found in mitosis: cytoplasmic microtubules disassemble at the onset of mitosis and reassemble into the mitotic spindle, which, in turn, disassembles at the end of mitosis when cytoplasmic microtubules are reformed. Lamellipodial activity and directional cell motility in many cell types also depends strongly on spatial and temporal regulation of microtubule dynamics [Bershadsky et al., 1991; Mikhailov and Gundersen, 1998; Elbaum et al., 1999].

At present, the information about the mechanisms responsible for overall coordination of microtubule dynamics is very restricted. Thus, treatments that affect microtubule dynamics by modulating specific signaling pathways are highly interesting.

In this study, we show that a specific group of tyrphostins from the nitro-benzene malononitrile family, namely, AG-1714, AG-1798, AG-1781, AG-1801, and AG-1820, induce microtubule disassembly in living cells. However, unlike essentially all known microtubule-disrupting drugs, these tyrphostins do not affect the polymerization or stability of microtubules in vitro. Microtubule disassembly in living cells induced by AG-1714, the most effective compound in the group, was blocked by taxol, which stabilizes microtubules, and pervanadate, which blocks tyrosine phosphatases and increases phosphorylation levels. These results suggest that tyrosine phosphorylation is involved in the regulation of microtubule dynamics.

MATERIALS AND METHODS

Cells

The cells used in this study include bovine aortic endothelium (BAE) cells (kindly provided by Dr. Israel Vlodavsky, The Hebrew University, of Jerusalem), SV80 cells, derived from SV40-transformed human fibroblasts [Kahn et al., 1983] and Swiss 3T3. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum (Hyclone Laboratories Inc., Logan, UT). Cells were split soon after reaching confluence by 2-min treatment with 0.1% trypsin. Rat hippocampal neurons (kindly provided by Prof. Anthony Futerman, Weizmann Institute of Science, Rehovot) were cultured as described [Goslin and Banker, 1990] with minor modifications [Harel and Futerman, 1993; Schwarz et al., 1995].

Tyrphostins

A family of tyrphostins with similar structure, AG-1714, 1798, 1781,1801, and 1820, were synthesized as previously described [Gazit et al., 1989]. Stock solutions (0.1M) were prepared in DMSO and kept at −20°C. Aliquots of the different tyrphostins were added to complete culture medium at final concentrations ranging from 0.1–100 μM.

Taxol and Pervanadate Treatment

Cells cultured on glass coverslips were treated with 20 μM taxol for 3 h and AG-1714 was added for an additional 30 min. The cells were washed and permeabilized-fixed (see below).

Cells were also washed in serum-free DMEM and treated for 3 min with pervanadate (1 mM sodium orthovanadate with 1 mM H_2O_2 in serum-free DMEM) and AG-1714 was added for an additional 10 min. The cells were washed, permeabilized-fixed, and processed for immunofluorescence microscopy labeling.

Indirect Immunofluorescence Labeling

For labeling of actin, phosphotyrosine and paxillin, cells cultured on glass coverslips were permeabilized and fixed for 2 min with a mixture of 0.5% Triton X-100 and 3% paraformaldehyde and further fixed for 20 min with 3% paraformaldehyde without detergent. For tubulin labeling, the cells were fixed with cold 100% methanol at −20°C for 10 min. The fixed cells were rinsed with PBS and incubated for 40 min at room temperature with the appropriate primary antibodies, washed 3 times with PBS, and incubated for 40 min with the fluorophore-conjugated secondary antibodies. The labeled coverslips were mounted with Elvanol (Mowiol 4-88, Hoechst, Frankfurt, Germany) and examined using a Zeiss Axiophot microscope with a ×100/1,3 Planapo objective.

Antibodies

Monoclonal anti-phosphotyrosine (PT-66) and monoclonal anti α-tubulin (DM 1A) were purchased from Sigma Immunochemicals (Rehovot, Israel), and monoclonal anti-paxillin was purchased from Transduction Labs (Lexington, KY). Actin filaments were visualized with TRITC-phalloidin (Sigma, St. Louis, MO) and Cy3-conjugated secondary antibodies were purchased from Jackson Labs (Bar Harbor, MI)

In Vitro Treatment With Tyrphostin AG-1714

Tubulin purified from bovine brain by two cycles of polymerization-depolymerization followed by passage over P-11 phosphocellulose (Whatman International, Maidstone, UK) to remove microtubule associated proteins (MAPs), and a final cycle of polymerization-depolymerization [Williams and Lee, 1982; Williams, 1992] was used for the in vitro experiments. Non-polymerized tubulin (~250 μg) was treated with either 0.1% DMSO, 10 μM nocodazole, or 100 μM tyrphostin AG-1714 and exposed to polymerization conditions (incubation at 30°C in 100 μl buffer containing 1 mM GTP, 1 mM EGTA, 2 mM $MgSO_4$ in 0.1M Pipes-NaOH buffer, pH 6.9). Aliquots of 20 μl were examined at indicated time points for the presence of microtubules in a Zeiss Axiovert microscope, using Nomarski DIC optics with a Fluar 100/1,3 objective. Images were recorded using a digital CCD video camera (iSight, Tirat Hacarmel, Israel) and enhanced by background correction and contrast amplification using Matrox Inspector software (Matrox, Dorval, Canada).

Cell Extract and Immunoblotting

Swiss 3T3 cells cultured in 30 mm tissue culture plates (Falcon, Plymouth, UK) were incubated in serum-free medium for 24–48 h prior to treatments. Tyrphostin solution was added to the cultured cells for 5–60 min, after which the cells were washed with cold PBS, scraped off using a rubber policeman, and extracted with sample buffer [Laemmli, 1970]. The samples were examined by SDS-PAGE (5–15% polyacrylamide gradient) under reducing conditions. The proteins were transferred to Hybond-C nitrocellulose paper (Amersham Life Science, Buckinghamshire, UK). Immunoblotting analysis was carried out by incubating O.N. at 4°C with anti phosphotyrosine antibodies (PT-66 diluted 1:5,000) then intensely washed with buffer containing 150 mM NaCl, 10 mM Tris-HCl, and 0.05% Tween 20, pH 7.6, and further incubated with HRP-conjugated goat anti-mouse immunoglobulin G (Amersham). The immunoreactive bands were visualized using the Enhanced Chemiluminiscence (ECL) method.

RESULTS

Effect of Nitro-Benzene Malononitrile Tyrphostins on Microtubule Stability

In a preliminary broad screen of several tyrphostins for their cytoskeletal effects we have identified a highly potent compound, AG-1714, which, upon addition to living BAE cells, induces microtubule disassembly (Fig. 1). Other tyrphostins included in the screen had different effects on the cytoskeleton including strong disruption of the actin-based microfilament system and membrane anchorage of focal contacts and cell-cell junctions. Other tyrphostins enhanced the assembly of cell-cell junctions without affecting microtubules (Volberg et al., unpublished data).

To explore the structural basis for this effect we examined additional compounds, structurally-related to AG-1714. Figure 1 shows that, within the group tested, AG-1714 had the most profound effect, and was more potent than AG-1781, which differs only in the position of the nitro group. Replacement of one of the CN groups with a substituted amide markedly reduces the potency of AG-1798 and AG-1801, while replacement of the CN with $H_2NC = (CN)_2$ rendered the compound AG-1820 essentially inactive. Since AG-1714 was the most potent member of the family, a detailed characterization of its effect was carried out.

The effect of AG-1714 on microtubules is both concentration- and time-dependent. As shown in Figure 2, microtubule disruption is apparent after 45 min treatment with 1–10 μM AG-1714. The effect was manifested by a decrease in the number and length of cytoplasmic microtubule and the appearance of diffuse

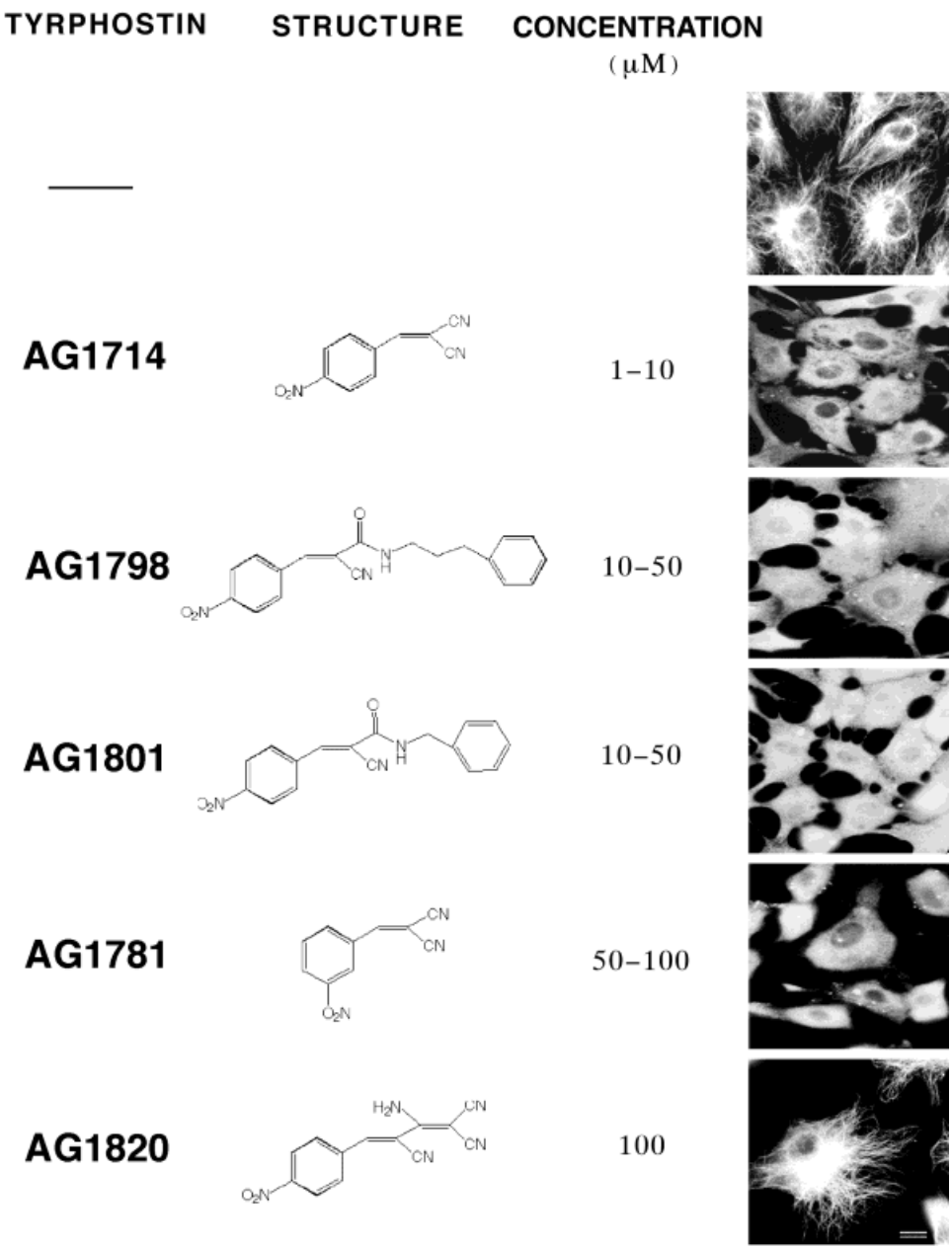

Fig. 1. The effect of structurally related tyrphostins on the microtubule network. Tyrphostins AG-1714, AG-1798, AG-1801, AG-1781, and AG-1820 all belong to the family of nitro-benzen malononitriles. Treatment of Bovine Aortic Endothelial (BAE) cells with these compounds, except AG-1820, induced disruption of the microtubular network within 45 min of incubation. The tyrphostins were effective in the concentration range of 1–100 μM, and AG-1714 was the most potent. Bar = 10 μm.

cytoplasmic tubulin staining (Fig. 2B,C). AG-1714 at 100 μM leads to complete disassembly of the microtubular network (Fig. 2D). At this concentration, microtubule disruption is complete within 10 min (not shown).

Similar effects of AG-1714 on the microtubular system in BAE cells were observed also in other cell types. As shown in Figure 3, for SV80 cells (Fig. 3A,B) and Swiss 3T3 cells (Fig. 3C,D), substantial microtubule disassembly leads to accumulation of diffuse tubulin staining throughout the cytoplasm subsequent to treatment with AG-1714. In neurits of untreated hippocampal neurons, dense arrays of microtubules are present and individual microtubules cannot be readily resolved. Following treatment with AG-1714, these microtubule bundles are severely affected and the neurits appear fragmented (Fig. 3E,F).

AG-1714 Induces Reorganization of Microfilaments and Staining Pattern for Phosphotyrosine

The effect of AG-1714 on the microtubular system is accompanied by major reorganization of the microfilament system and the phosphotyrosine-containing focal adhesions. In untreated BAE endothelial cells, actin filaments are assembled into short bundles (Fig. 4A) and

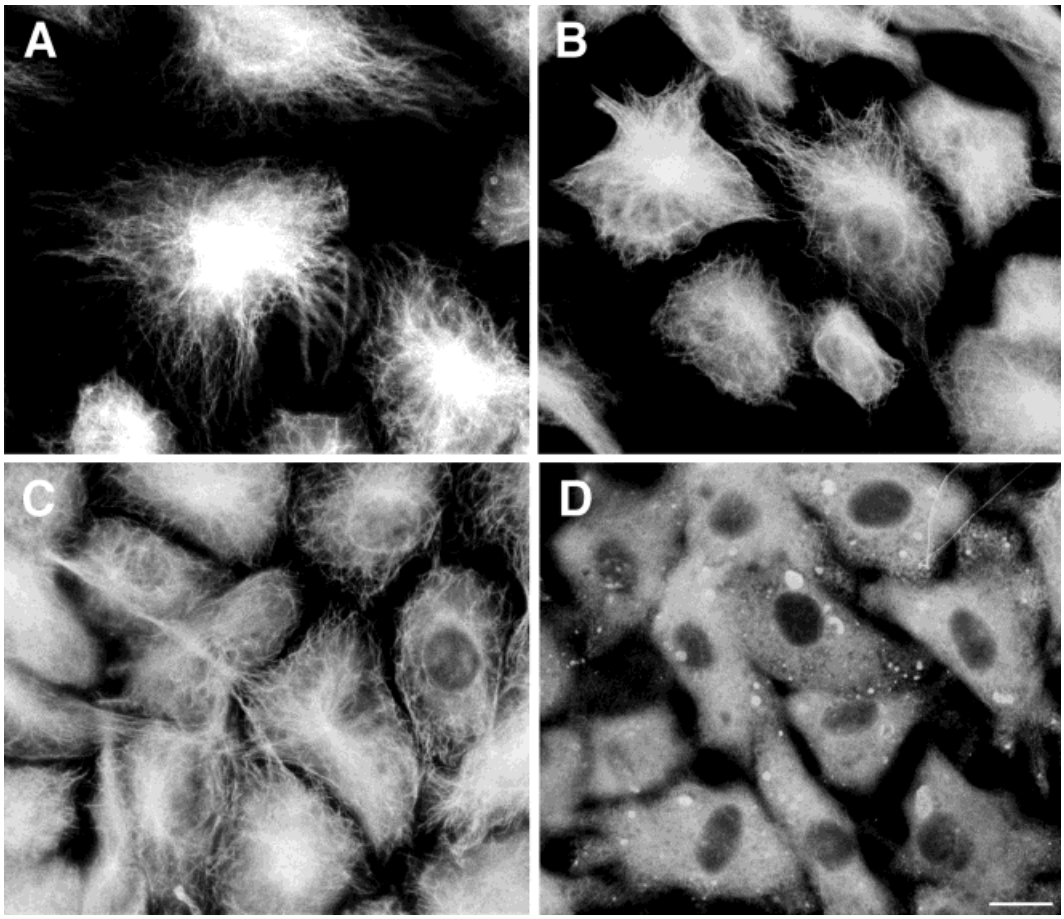

Fig. 2. The effect of tyrphostin AG-1714 on MT network. BAE cells were treated with 0 (**A**), 1 (**B**), 10 (**C**), and 100 (**D**) μM AG-1714 for 45 min. The cells were permeabilized/fixed and labeled with monoclonal antibody against α-tubulin. The effect of AG-1714 was noticeable even at 1 μM and the MT network was totally disrupted at 100 μM. Bar = 10 μm.

phosphotyrosine labeling is specifically associated with cell-cell adherens junctions (Fig. 4B). Following AG-1714 treatment, actin organization is dramatically transformed into densely packed stress fibers (Fig. 4C) and phosphotyrosine labeling that is initially associated mainly with cell-cell junctions, is greatly enhanced, and is primarily associated with focal contacts (Fig. 4D). Similar effects on the microfilament system and focal contacts have also been obtained by microtubule depolymerization with nocodazole or other microtubule-disrupting drugs [Bershadsky et al., 1996].

Disassembly of Microtubules by AG-1714 Is Blocked by the Microtubule-Stabilizing Agent, Taxol, or by the Tyrosine Phosphatase Inhibitor, Pervanadate

The addition of 20 μM taxol to cells, 3 h prior to addition of 100 μM AG-1714 for an additional 30 min, completely blocks microtubule disruption. In taxol-treated cells, microtubules form tight bundles that are essentially resistant to disruption by AG-1714 (Fig. 5C,D). It is noteworthy that taxol can markedly stabilize microtubules against disruption with a variety of drugs such as nocodazole [Bershadsky et al., 1996]. The disruptive effect of AG-1714 on microtubules could also be blocked by short treatment (13 min) of the cells with 1 mM pervanadate (Fig. 5E,F). Such treatment was previously shown to dramatically increase the levels of phosphotyrosine in cells due to the inhibition of tyrosine-specific phosphatases [Volberg et al., 1992].

Tyrphostin AG-1714 Does Not Affect Microtubule Assembly In Vitro

To determine whether tyrphostin AG-1714 directly interferes with microtubule stability, its effect on microtubule formation and stability in vitro was examined. For this purpose AG-1714 (100 μM) was added to purified

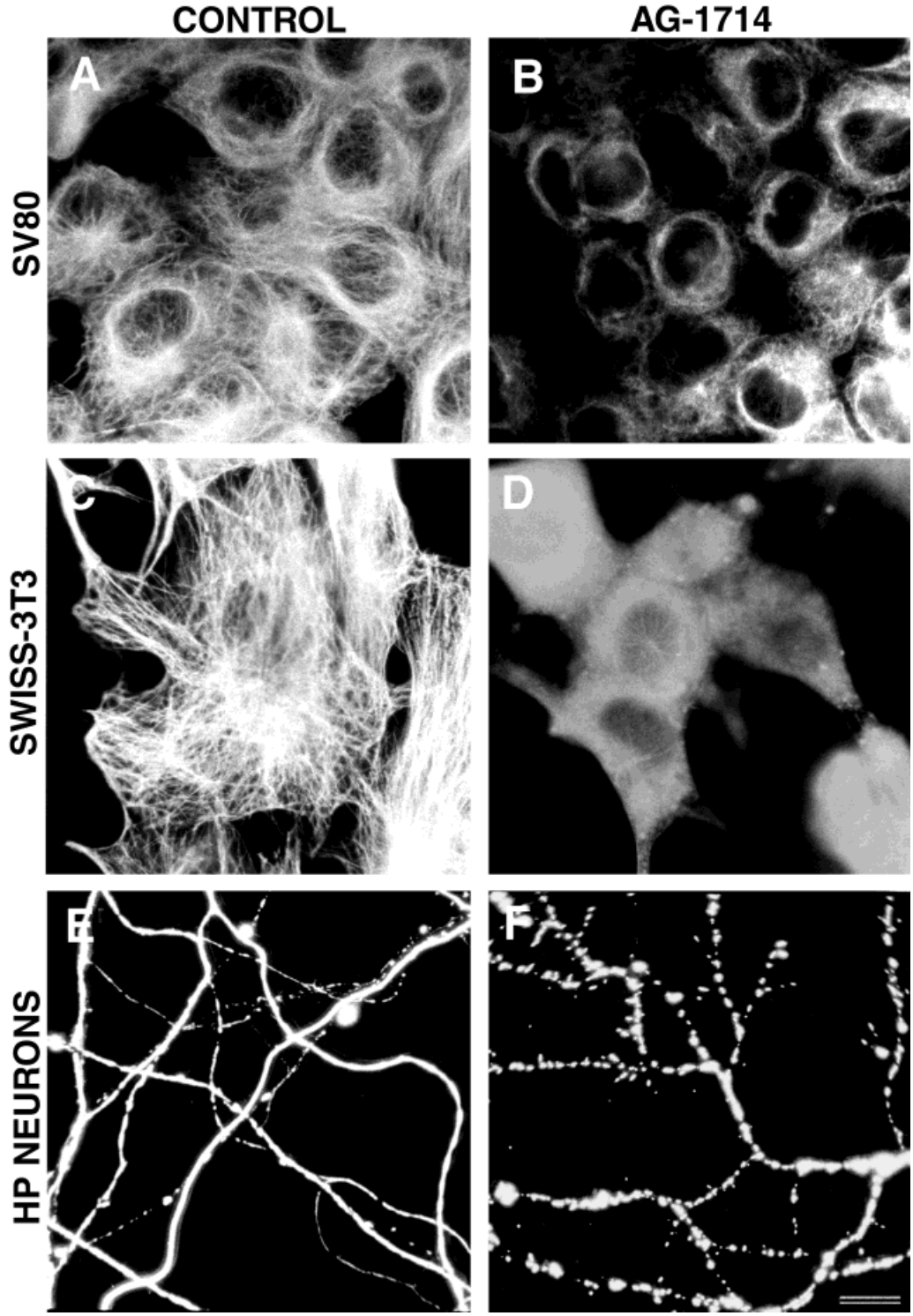

Fig. 3. AG-1714 disrupts MT in different cell types. SV80 human fibroblasts (**A,B**), Swiss 3T3 mouse fibroblasts (**C,D**), and rat hippocampal neurons (**E,F**) were treated with 100 μM AG-1714 (B,D,F) for 30 min. AG-1714 almost totally disrupted the MT network in SV80 and Swiss 3T3 cells, and caused characteristic fragmentation of microtubule bundles in the neurites of rat hippocampal cells. Bar = 10 μm.

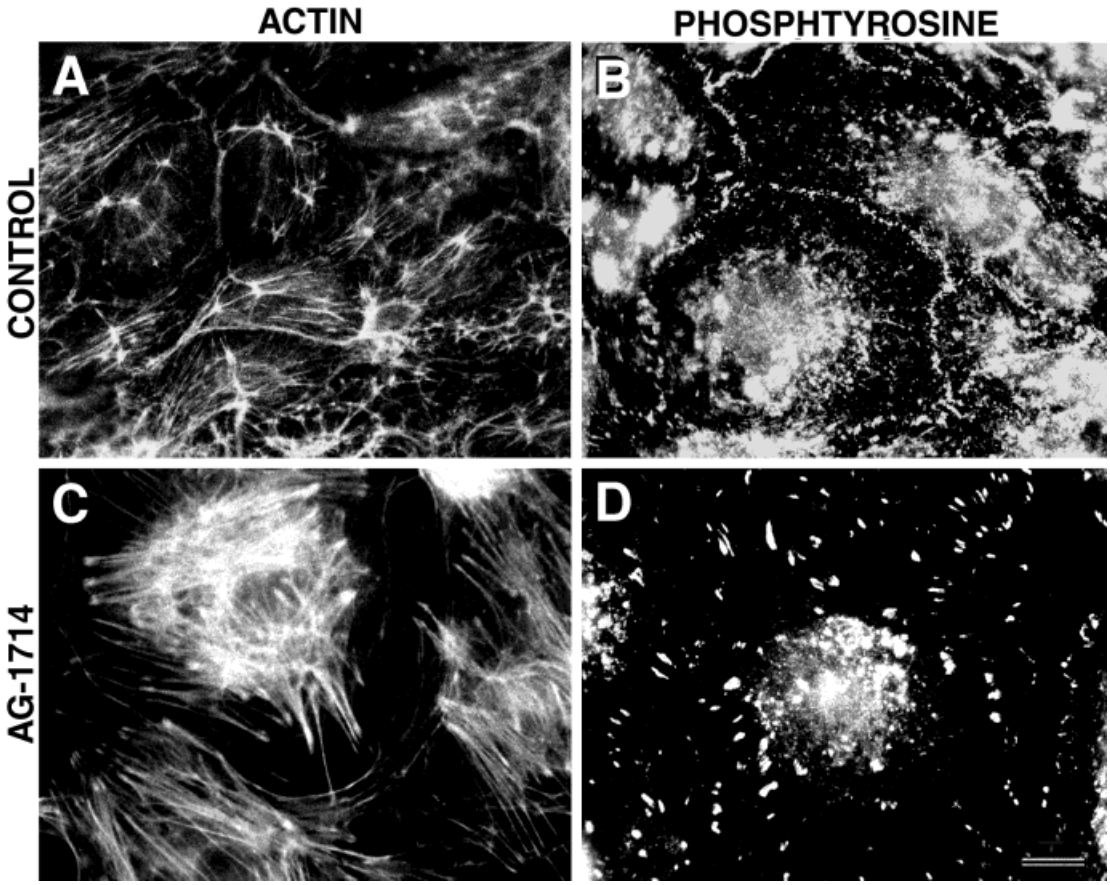

Fig. 4. AG-1714 induces reorganization of actin cytoskeleton and alters the tyrosine phosphorylation pattern. Non-treated BAE cells (**A,B**) and cells treated with 100 μM AG-1714 for 45 min (**C,D**) were permeabilized/fixed and labeled with rhodamine-conjugated phalloidin (A,C) and monoclonal antibody against phosphotyrosine (B,D). Note formation of prominent stress fibers (C) and phosphotyrosine-positive focal contacts (D) in AG-1714-treated cells. Bar = 10 μm.

tubulin solution under polymerization conditions. At 30, 60, and 120 min of incubation, aliquots were examined by Nomarski DIC optics for the presence of long microtubules. As shown in Figure 6, the control sample ("DMSO") contains numerous microtubules, whereas the sample treated with nocodazole is devoid of visible microtubules (Fig. 6, "nocodazole" middle column). Addition of AG-1714 to the polymerization solution has no apparent effect on the density and length of microtubules.

Effect of AG-1714 on Tyrosine Phosphorylation Pattern

To determine the effect of tyrphostin AG-1714 on specific tyrosine phosphorylation events, we treated Swiss 3T3 cells for 0, 5, 15, 30, 45, and 60 min and compared the pattern of tyrosine phosphorylated proteins to those of untreated cells.

The effect of AG-1714 on tyrosine phosphorylation of specific proteins was quite complex as evident from Figure 7. Phosphorylation of several bands (notably 125 and 68 kDa bands corresponding to focal adhesion kinase [FAK] and paxillin) followed AG-1714 treatment. A similar effect was previously reported for cells treated with nocodazole [Bershadsky et al., 1996]. The stimulatory effect of AG-1714 on tyrosine phosphorylation was most prominent at 15 min after drug addition and then gradually declined. The intensity of some other bands (notably p98 and p92) decreased already in the first 5–15 min. At 60 min incubation with the drug, the phosphorylation levels of the major proteins bands were lower than in the control sample.

In the presence of taxol, when the microtubule-disrupting effect of AG-1714 was suppressed, the inhibitory effect of AG-1714 on tyrosine phosphorylation was more pronounced (Fig. 8). Thus, phosphorylation of some bands (p125, p98) after 1-h incubation with AG-1714 was apparently weaker in taxol-treated than in non-treated cells, and indeed weaker than in control cells and cells treated with taxol only.

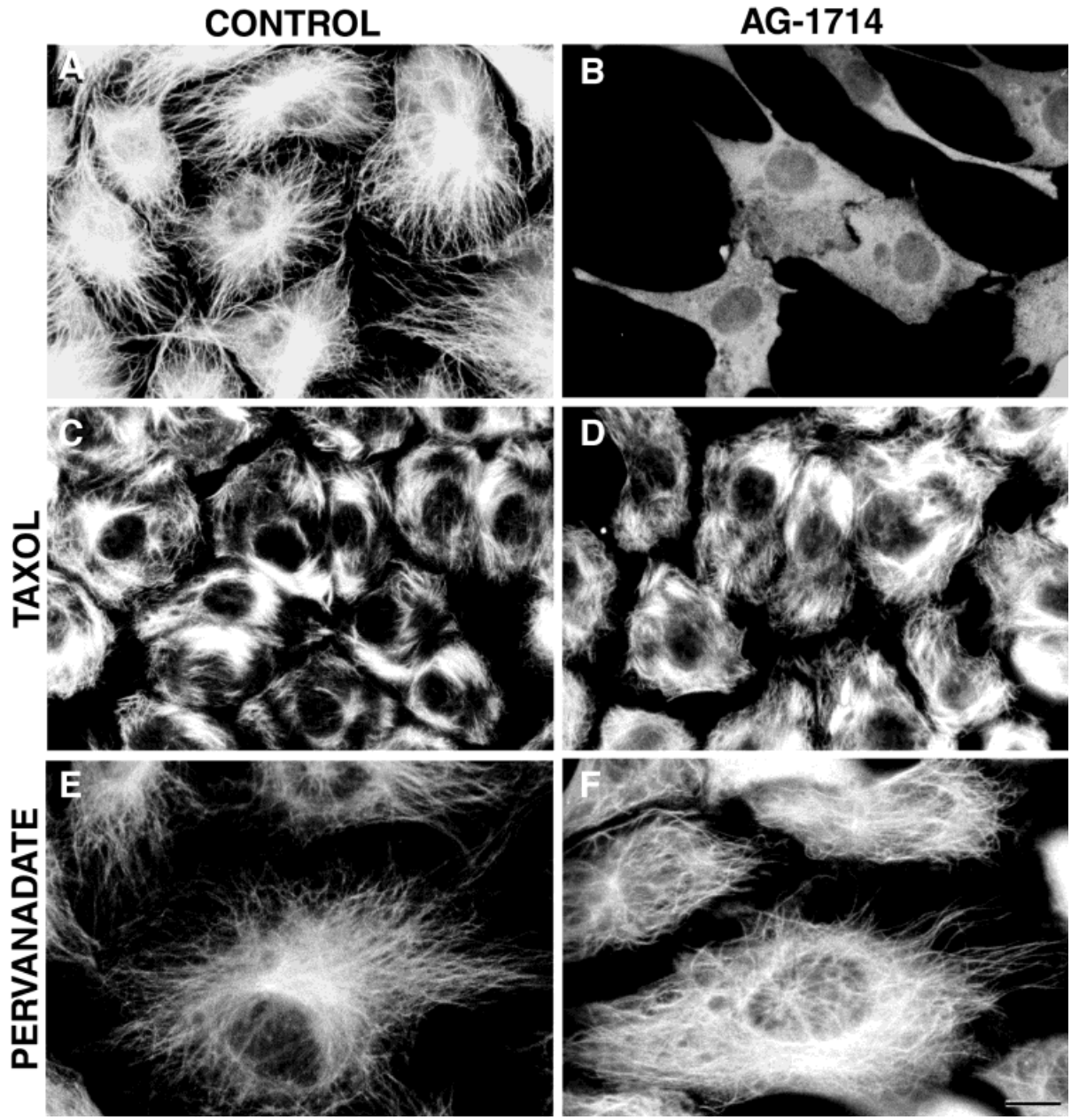

Fig. 5. Taxol and pervanadate (HV) prevent disruption of MT network by AG-1714. BAE cells were pretreated with 20 μM taxol for 3 h (**C**), or with 1mM pervanadate (HV) for 3 min (**E**), or left untreated (**A**); AG-1714 100 μM was then added to the taxol-treated sample for additional 30 min (**D**) and to the pervanadate-treated sample for additional 10 min (**F**). Control non-treated cells were treated with AG-1714 for 10 min (**B**) or for 30 min (not shown). The cells were permeabilized/fixed and labeled with monoclonal antibodies against α-tubulin. It is shown that both Taxol and HV prevented the disrupting effect of AG-1714 (compare D and F with B). Bar = 10 μm.

DISCUSSION

We report here on the capacity of a specific tyrphostin with the general structure of nitro-benzene malononitrile to disrupt microtubules in cultured cells. Unlike microtubule-disrupting drugs known to date, AG-1714 tyrphostins do not significantly affect polymerization of purified tubulin or microtubule stability in vitro.

Classical microtubule-disrupting or microtubule-stabilizing drugs such as colchicine, vinblastine, nocodazole, or taxol, bind to specific sites on the tubulin molecule and directly affect microtubule assembly-disassembly [Wilson and Jordan, 1995; Jordan and Wilson, 1998]. Tyrphostin AG-1714, on the other hand, induces microtubule depolymerization indirectly, most probably

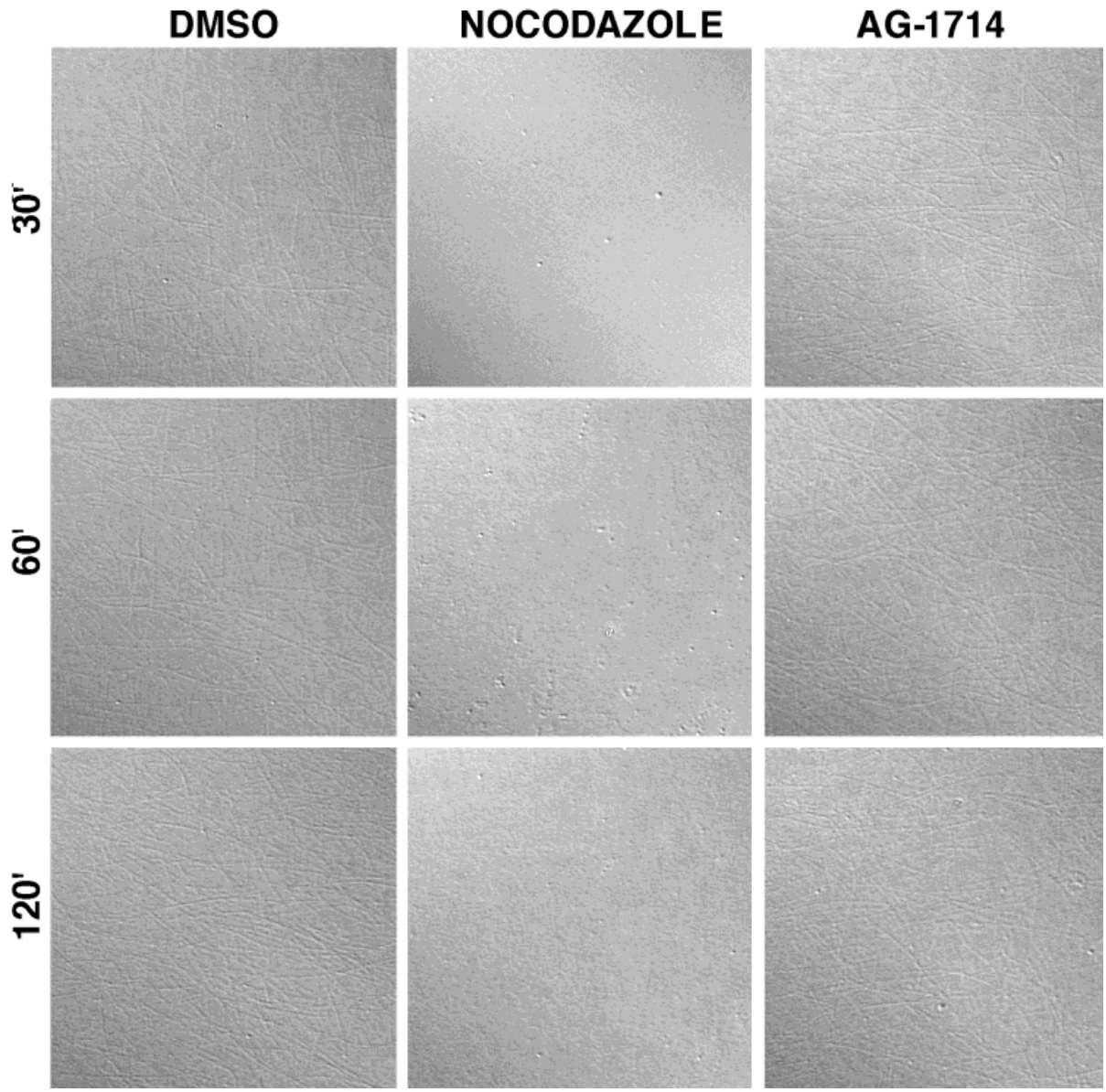

Fig. 6. AG-1714 does not affect MT polymerization in vitro. Phosphocellulose-purified tubulin (250 μg in 100 μl) was allowed to polymerize at 30°C in the presence of 1 mM GTP. The samples were observed using DIC optics; the photographs represent typical fields at different time intervals. Addition of 100 μM AG-1714 did not affect the polymerization level as compared with the control sample to which 0.1% DMSO was added in order to equalize its concentration in the three sample types. Nocodazole (10 μM) completely prevented tubulin polymerization.

via its effect on a regulatory system controlling microtubule stability.

The only known agent that also disrupts microtubules in vivo but does not inhibit tubulin polymerization in vitro is a potent uncoupler of oxidative phosphorylation, FCCP [Maro et al., 1982]. It acts to decrease the cellular ATP level and may affect indirectly the balance between phosphorylation-dephosphorylation processes. However, decrease of the ATP level by other inhibitors led to stabilization rather than destabilization of cytoplasmic microtubules [Bershadsky and Gelfand, 1981], and unlike AG-1714, the application of FCCP was also detrimental to the microfilament system.

The evidence that the effect of AG-1714 on microtubule integrity is indeed attributable to the inhibition of specific tyrosine kinase(s) is compelling, but still indirect. The main support for such a mechanism is the result of the experiment with pervanadate, a potent broad specificity inhibitor of tyrosine phosphatases, which stabilizes cytoplasmic microtubules against AG-1714-mediated

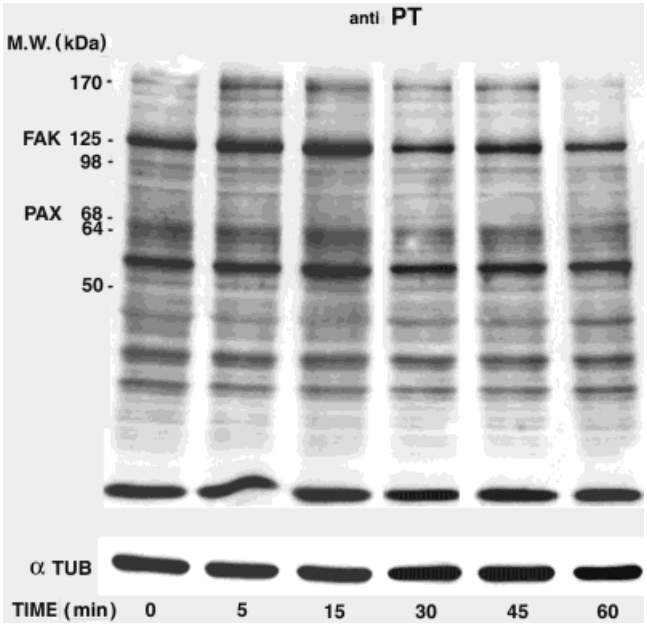

Fig. 7. The effect of AG-1714 on protein tyrosine phosphorylation. Swiss 3T3 cells were serum-starved for 48 h and treated with 100 μM AG-1714 for 5–60 min intervals. The samples were extracted with sample buffer, resolved on 5–15% gradient SDS PAGE, and immunoblotted with anti-phosphotyrosine antibody. Positions corresponding to FAK and paxillin are indicated.

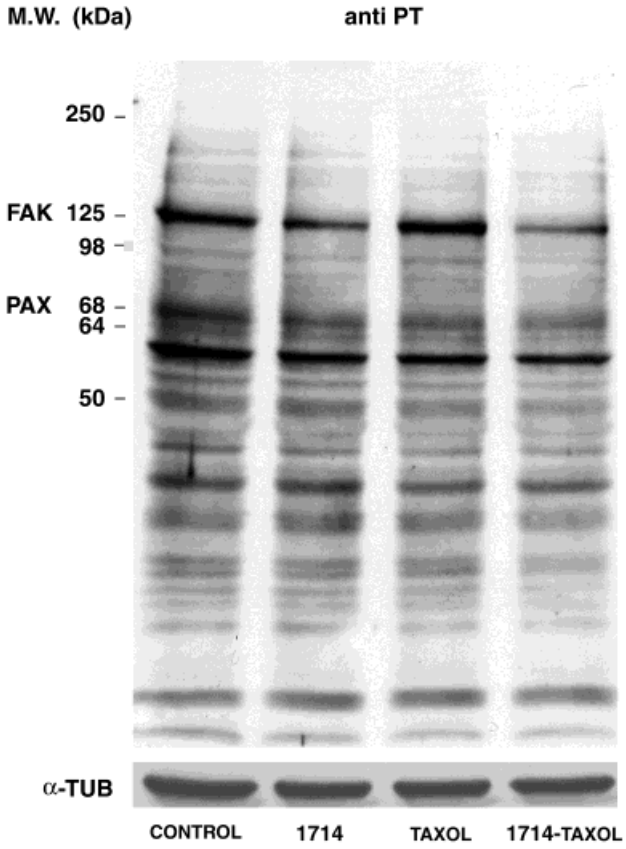

Fig. 8. The effect of Taxol on the AG-1714 induced changes in protein tyrosine phosphorylation. Tyrosine phosphorylation profiles of control and taxol-pretreated cells treated or non-treated with AG-1714 are presented. Duration of taxol pretreatment was 3 h; AG-1714 was added to taxol-containing medium and incubated for an additional 60 min.

disruption. Similar pervanadate treatment had no stabilizing effect against nocodazole-mediated disruption of microtubules (not shown). Pervanadate also does not apparently affect microtubules by itself. Thus, the effect of AG-1714 seems to be related to its capacity to inhibit tyrosine phosphorylation. AG-1714 may interfere with yet unidentified tyrosine phosphorylation-dependent signaling pathways that affect microtubule dynamics.

In an attempt to identify potential targets for AG-1714 we have used immunoblotting analysis of AG-1714-treated cells with anti-phosphotyrosine-specific antibody. The examination of the levels of tyrosine phosphorylated bands following AG-1714 treatment pointed to a highly complex effect. Many protein bands showed a transient increase in phosphotyrosine level reaching a peak at 5–15 min. The stimulation of tyrosine phosphorylation was most prominent in proteins such as FAK and paxillin. This apparently paradoxical increase in the tyrosine phosphorylation level induced by a putative tyrosine kinase inhibitor can be explained by the established capacity of microtubule disruption to induce an increase in protein tyrosine phosphorylation via activation of adhesion-dependent signaling [Bershadsky et al., 1996]. In fact, the specific proteins whose tyrosine phosphorylation increased in response to AG-1714 treatment were similar to those whose tyrosine phosphorylation increased in response to nocodazole or vinblastine [Bershadsky et al., 1996]. This implies that the observed alteration of tyrosine phosphorylation of FAK, paxillin, and other proteins in response to AG-1714 might be a consequence of microtubule disruption rather than a result of direct stimulatory effect of AG-1714 on the phosphorylation of these proteins.

It should be pointed out that AG-1714 did induce additional changes in the level of tyrosine phosphorylation of several protein bands (e.g., decrease of the phosphorylation of p98 and p92 in the first 15 min of treatment), yet the significance of those changes is not clear. Moreover, we do not know whether a decrease in phosphorylation of several bands induced by AG-1714 in the presence of taxol, when microtubules remain intact (Fig. 8), is related to its effect on microtubules. It is possible that proteins involved in microtubule destabilization, whose tyrosine phosphorylation is affected by AG-1714, are not abundant in the

cell and thus avoid detection, or that changes in their phosphorylation induced by AG-1714, are blocked by taxol. Finally, we cannot exclude the possibility that the effect of AG-1714 on microtubules does not involve inhibition of tyrosine phosphorylation despite the indirect evidence derived from the pervanadate effect, and that the stabilization of microtubules is not related to the inhibitory effect of vanadate on phosphotyrosine phosphatases.

What might be the cellular targets of AG-1714 that are relevant to its effect on microtubules? In general, the state of microtubules in the cell depends on a balance between the activity of stabilizing and destabilizing factors. Factors of both types were shown to be strictly regulated and tyrosine phosphorylation could be involved in such regulation. For example, classical MAPs are known to be potent stabilizers of microtubules [Mandelkow and Mandelkow, 1995]. However, serine/threonine phosphorylation of these molecules by MT-affinity-regulating kinases (MARKs) induces their detachment from microtubules leading to microtubule depolymerization [Drewes et al., 1998]. The activity of MARKs, in turn, can in principle be regulated by phosphorylation on serine, threonine, and tyrosine residues [Drewes et al., 1998]. Tau, a neural microtubule-associated protein important in axonal development, was shown to interact directly with the SH3 domains of the cytoplasmic tyrosine kinases, fyn and src, and to undergo tyrosine phosphorylation [Lee et al., 1998].

Among the factors promoting microtubule depolymerization, stathmin/Op18 seems to be most effective [Lawler, 1998]. Activity of this protein is negatively regulated by phosphorylation on four serine residues, which is stimulated by a wide range of external effectors [Lawler, 1998; Andersen, 1999]. Tyrosine phosphorylation events (induced, for example, by nerve growth factor) may regulate stathmin activity [Di Paolo et al., 1996].

Several studies indicate that alpha-tubulin itself can be phosphorylated on tyrosine residues present within an acidic stretch of amino acids located near the carboxy terminus [Peters et al., 1996]. The cytoplasmic protein kinase Syk as well as insulin receptor kinase were shown to be responsible for this phosphorylation [Wandosell et al., 1987; Peters et al., 1996]. The significance of alpha-tubulin tyrosine phosphorylation in the regulation of microtubule dynamics is still unclear.

Finally, since the mechanisms of AG-1714 action on microtubule dynamics are most likely indirect, it is important to keep in mind that this molecule may also affect the activity of microtubule-associated proteins that do not undergo tyrosine phosphorylation.

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