

# Mechanisms by which Synthetic 6,7-Annulated-4-substituted Indole Compounds with Anti-proliferative Activity Disrupt Mitosis and Block Cytokinesis in Human HL-60 Tumor Cells *In Vitro*

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**Abstract.** *Background:* Synthetic 6,7-annulated-4-substituted indole compounds, which elicit interesting antitumor effects in murine L1210 leukemia cells, were tested for their ability to inhibit human HL-60 tumor cell proliferation, disrupt mitosis and cytokinesis, and interfere with tubulin and actin polymerization *in vitro*. *Materials and Methods:* Various markers of metabolic activity, mitotic disruption and cytokinesis were used to assess the effectiveness of the drugs in the HL-60 tumor cell system. The ability of annulated indoles to alter the polymerizations of purified tubulin and actin were monitored in cell-free assays and were compared to the effects of drugs known to disrupt the dynamic structures of the mitotic spindle and cleavage furrow. *Results:* With one exception, annulated indoles inhibited the metabolic activity of HL-60 tumor cells in the low-micromolar range after two and four days in culture but these anti-proliferative effects were weaker than those of jasplakinolide, a known actin binder that blocks cytokinesis. After 24-48 h, antiproliferative concentrations of annulated indoles increased the mitotic index of HL-60 cells similarly to vincristine and stimulated the formation of many

*bi-nucleated cells, multi-nucleated cells and micronuclei, similarly to taxol and jasplakinolide, suggesting that these antitumor compounds might increase mitotic abnormality, induce chromosomal damage or missegregation, and block cytokinesis. Since annulated indoles mimicked the effect of vincristine on tubulin polymerization, but not that of taxol, these compounds might represent a new class of microtubule de-stabilizing agents that inhibit tubulin polymerization. Moreover, annulated indoles remarkably increased the rate and level of actin polymerization similarly to jasplakinolide, suggesting that they might also stabilize the cleavage furrow to block cytokinesis. Conclusion: Although novel derivatives with different substitutions must be synthesized to elucidate structure-activity relationships, identify more potent antitumor compounds and investigate different molecular targets, annulated indoles appear to interact with both tubulin to reduce microtubule assembly and actin to block cytokinesis, thereby inducing bi- and multinucleation, resulting in genomic instability and apoptosis.*

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Indole arynes, or indolynes, represent a new class of reactive aryne intermediate (1-7) but the biological properties of annulated indoles have not been fully-evaluated and exploited. However, a few biologically-active natural products such as trikenetrins, herbindoies, teleocidins and nodulisporic acids have been reported (8-11), suggesting that annulated indoles may have unique structural features and a good probability of exhibiting interesting biological activity (12). Because annulated indoles have almost no representation in the NIH PubChem and Molecular Library Small Molecule Repository (MLSMR) databases, an unprecedented class of

an indole-based library was constructed, using the indole aryne methodology (12), and screened for antitumor activity (13). Hence, 66 novel 6,7-annulated-4-substituted indole compounds were synthesized, using a strategic combination of 6,7-indolyne cycloaddition and cross-coupling reactions under both Suzuki-Miyaura and Buchwald-Hartwig conditions (12), and tested for their effectiveness against murine L1210 tumor cell proliferation *in vitro* (13). Most compounds inhibited the metabolic activity of L1210 lymphocytic leukemia cells in a time- and concentration-dependent manner but only nine of them were sufficiently potent to inhibit L1210 tumor cell proliferation by 50% in the low micromolar range after two [concentration inhibiting by 50% ( $IC_{50}$ )=4.5-20.4  $\mu$ M] and four days [ $IC_{50}$ =0.5-4.0  $\mu$ M] in culture (13). A 3-h treatment with antiproliferative annulated indole was sufficient to inhibit, in a concentration-dependent manner, the rate of DNA synthesis measured in L1210 cells over a 0.5-h period of pulse-labeling with  $^3$ H-thymidine (13). Four of the antiproliferative compounds had weak DNA-binding activities but one compound reduced the fluorescence of the ethidium bromide-DNA complex by up to 53%, suggesting that some annulated indoles might directly interact with double-stranded DNA to disrupt its integrity and prevent the dye from intercalating into DNA base pairs (13). However, all nine antiproliferative compounds induced DNA cleavage at 24 h in L1210 cells that contained  $^3$ H-thymidine-prelabeled DNA, suggesting that these antitumor-annulated indoles might trigger an apoptotic pathway of DNA fragmentation (13). Indeed, these annulated indoles caused a time-dependent increase of caspase-3 activity with a peak at 6 h (13). Interestingly, antiproliferative concentrations of annulated indoles increased the mitotic index of L1210 cells and stimulated the formation of many bi-nucleated cells (BNCs), multi-nucleated cells (MNCs), apoptotic cells and micronuclei (MNI) after 24-48 h, suggesting that these antitumor compounds might increase mitotic abnormality, induce chromosomal damage or missegregation, and block cytokinesis to induce apoptosis (13). Therefore, the present study was undertaken to determine the effectiveness of the six most potent annulated indoles against human HL-60 tumor cell proliferation, assess their ability to alter the kinetics of tubulin and actin polymerization, and compare their effects to those of drugs known to interact with microtubules (MTs) and actin filaments in order to disrupt the functions of the mitotic spindle and cleavage furrow.

## Materials and Methods

**Drug treatment, cell culture and proliferation assay.** The synthesis of 6,7-annulated-4-substituted indoles, using a strategic combination of 6,7-indolyne cycloaddition and cross-coupling reactions under both Suzuki-Miyaura and Buchwald-Hartwig conditions, may represent the first example of library development that employs the indole aryne methodology (12). The four steps of the synthesis

process, showing cycloaddition at the 6,7 position, followed by cross-coupling at the C4 position have been summarized elsewhere (13). Solutions of synthetic 6,7-annulated-4-substituted indoles, known MT-disrupting anticancer drugs used as positive controls, vincristine (VCR) and taxol (all from Sigma-Aldrich, St. Louis, MO, USA), and the actin binder jasplakinolide (JAS; Calbiochem, EMD Millipore, Billerica, MA, USA) were dissolved and serially diluted in dimethyl sulfoxide (DMSO). Suspension cultures of human HL-60 promyelocytic leukemia cells (American Type Culture Collection, Manassas, VA, USA) were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and maintained in continuous exponential growth by twice-a-week passage in RPMI-1640 medium supplemented with 10% fetal bovine calf serum (FCS; Atlanta Biologicals, Norcross, GA, USA) and penicillin (100 IU/ml)-streptomycin (100  $\mu$ g/ml). HL-60 cell suspensions were grown in triplicate in 48-well Costar cell culture plates for two and four days in the presence or absence (control) of serial concentrations of synthetic 6,7-annulated-4-substituted indoles to evaluate their antiproliferative activity. Since compounds were supplemented to the culture medium in 1- $\mu$ l aliquots, the concentrations of vehicle in the final incubation volume (0.5 ml) never exceeded 0.2% and did not interfere with the data. Decreasing densities of cells, such as 125,000 and 37,500 HL-60 cells/0.5 ml/well, were initially plated in triplicate at time 0 in order to collect control samples with approximately equal cell densities after two and four days in culture, respectively. The proliferation of control and drug-treated tumor cells was assessed from their mitochondrial ability to bioreduce the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, Madison, WI, USA) in the presence of phenazine methosulfate (PMS; Sigma) into a water-soluble formazan product that absorbs at 490 nm (14). After two or four days in culture, control and drug-treated HL-60 cell samples were further incubated at 37°C for 2.5 h in the dark in the presence of 0.1 ml of MTS:PMS (2:0.1) and their relative metabolic activity was estimated by recording the absorbance at 490 nm, using a Cambridge model 750 automatic microplate reader (Packard, Downers Grove, IL, USA) (13). Blank values for culture medium supplemented with MTS:PMS reagent in the absence of cells were subtracted from the results. Data of all biochemical experiments were analyzed using Student's *t*-test with the level of significance set at  $p < 0.05$ .

**Mitotic index and abnormalities.** To determine the mitotic index, HL-60 cells ( $0.5 \times 10^6/0.5$  ml of FCS-containing RPMI-1640 medium) were incubated in triplicate for various periods of time at 37°C in the presence or absence (control) of serial concentrations of experimental drugs or known MT-disrupting and actin-stabilizing agents, collected by centrifugation (200  $g \times 10$  min), and re-suspended in 1 ml of hypotonic 75 mM KCl for 20 min at 4°C. After fixation in 1 ml of MeOH:acetic acid (3:1), the final cell pellets were collected by centrifugation, re-suspended in 75  $\mu$ l of MeOH:acetic acid (3:1), dispensed onto glass slides, air dried, and stained by spreading 40  $\mu$ l of 0.1% crystal violet under a coverslip (13). Mitotic figures with condensed chromosomes were identified microscopically and published criteria were followed to score BNCs, MNCs and MNI (15). Cytokinesis-blocked BNCs contained either two separate nuclei of equal size, two nuclei that touched or overlapped with distinct nuclear boundaries, or two nuclei that were linked by a small nucleoplasmic bridge (NPB) (15). Viable cells with three (trinucleated) or four (quadrinucleated) distinct nuclei

were scored as typical MNCs (15). Dot-like chromatin-containing structures in the cytoplasm, at least one-third smaller than the main nucleus, surrounded by a membrane either separated from or marginally overlapping the main nucleus, and having the same staining as the main nucleus were scored as MNi containing either a whole chromosome or an acentric chromosomal fragment (15). The frequencies of BNCs, MNCs and MNi were used to estimate the ability of cytotoxic or genotoxic (mutagenic or clastogenic) drugs to block cytokinesis and induce chromosomal damage or missegregation (15). The percentage of cells in mitosis or with two nuclei, MNCs and MNi were determined microscopically by counting a total of at least 4,000 cells/slide and the mitotic, BNC and MNC indexes were calculated as percentage of mitotic figures, BNCs, or MNCs in drug-treated cultures relative to that in vehicle-treated controls (13, 15).

**Tubulin polymerization assay.** The polymerization of purified tubulin protein from bovine brain in the presence and absence of glycerol was analyzed in a cell-free system using the Tubulin/Microtubule Biochem Kit purchased from Cytoskeleton (Denver, CO, USA) (16-18). The polymerization reactions contained, in a final volume of 0.2 ml, either tubulin without glycerol (1.15 mg/ml) or tubulin plus 15% glycerol (2.25 mg/ml) in 80 mM PIPES buffer, pH 6.9, supplemented with 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM GTP and either 0 or 15% glycerol. The annulated indoles and known MT-disrupting or actin-stabilizing agents under study were all added to the assay mixture in 1- $\mu$ l aliquots of DMSO to obtain the final concentrations tested. This vehicle did not affect the kinetics of tubulin polymerization in drug-untreated control reactions incubated in the presence or absence of glycerol. The turbidity assay mixtures were immediately incubated at 35°C in quartz microcells and the rate and plateau of tubulin polymerization were continuously monitored by scanning and recording over 900 s the increased absorbance of the solution at 340 nm, using a Shimadzu UV-160 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with dual-beam optics and a thermostatically-controlled cell holder (17, 18).

**Actin polymerization assay.** The purified actin protein from rabbit skeletal muscle provided in the Actin Polymerization Biochem Kit purchased from Cytoskeleton was used to analyze in a cell-free system the polymerization of globular-actin (G-actin) monomers to form long filamentous-actin (F-actin) with the concomitant hydrolysis of ATP in the presence of divalent cations. There is a critical concentration (CC) of free G-actin monomers below which actin will not polymerize. At monomer concentrations above the CC, actin will polymerize until the remaining free G-actin monomer concentration becomes equal to the CC. The presence of Mg<sup>2+</sup> and KCl lowers the CC to induce nucleation and Mg<sup>2+</sup> also stabilizes F-actin. *N*-(1-Pyrenyl)iodoacetamide is a fluorophore that binds to the thiol group of the Cys 374 residue of the G-actin monomers. Because the very weak fluorescence of the pyrene-labeled G-actin is dramatically enhanced upon incorporation into the double-helical structure of F-actin filaments, the rate of actin polymerization in the absence (control), and presence of annulated indoles and known actin-stabilizing agent was monitored for 36 min at room temperature by recording the increased fluorescence (365 nm excitation and 407 nm emission) of pyrene-conjugated actin every 30 s-3 min (19, 20). The polymerization reactions contained, in a final volume of 0.2 ml, 0.45 mg/ml of pyrene-labeled actin in 10 mM Tris-HCl buffer, pH 7.5, supplemented with 0.2 mM CaCl<sub>2</sub>, 50 mM

KCl, 2 mM MgCl<sub>2</sub>, 5 mM guanidine carbonate and 1 mM ATP to lower the CC and initiate the polymerization reaction, were placed in 96-well Costar opaque assay plates and monitored, using a Cary Eclipse Fluorescence Spectrophotometer equipped with a microplate reader accessory (Varian, Walnut Creek, CA, USA). Data points were the means of replicates from three different experiments and the baseline fluorescence of pyrene-labeled G-actin in the absence of polymerization-inducing buffer was subtracted from the results.

## Results

**Inhibition of tumor cell proliferation.** Six of the synthetic 6,7-annulated-4-substituted indole compounds reported to have interesting antitumor effects in the L1210 tumor cell system (13) were selected for the present study with human HL-60 pro-myelocytic leukemia cells and their chemical structures are shown in Figure 1. For simplification, the full University of Kansas compound (KUC) identification numbers of these compounds synthesized at the NIH Center of Excellence in Chemical Methodologies and Library Development at the University of Kansas (KU-CMLD) have been logically abbreviated in our publications (13): for example, KUC107070 is KU-70, KUC107072 is KU-72, *et. al.* (Figure 1). All six compounds tested in the HL-60 tumor cell system had anti-proliferative activities in the micromolar range and their concentrations required to inhibit the mitochondrial ability of HL-60 cells to metabolize the MTS:PMS reagent by 50% after two and four days in culture are indicated in Table I, except for KU-191 which did not inhibit HL-60 cell proliferation by more than 34% and 48% at days 2 and 4, respectively. These anti-proliferative IC<sub>50</sub> values are always lower at day 4 than at day 2 because the magnitude of inhibition between the control cells that keep growing exponentially and the drug-treated cells that are inhibited continues to widen over time (Table I). Full concentration-response curves indicate that the time-dependent inhibitions of HL-60 tumor cell growth by antiproliferative KU-70, KU-72 and KU-80 at days 2 and 4 begin above 4  $\mu$ M and become maximal or near-maximal around 25-62.5  $\mu$ M (Figure 2). However, when tested as positive controls in the same experiments, established anticancer drugs like daunorubicin and mitoxantrone (data not shown), and JAS (Table I and Figure 2), a cell-permeable F-actin probe with fungicidal, insecticidal and antitumor properties, inhibited the growth of HL-60 tumor cells at much lower nanomolar concentrations.

**Mitotic index and abnormalities.** Microscopic studies were conducted to determine whether HL-60 tumor cells might undergo mitotic disruptions after one and two days when treated with 10- $\mu$ M annulated indoles, which began to elicit antiproliferative activities at day 2 (Figure 2). Control populations of HL-60 tumor cells incubated in the absence of drugs contained only 1.34% of mitotic cells and 2.66% of

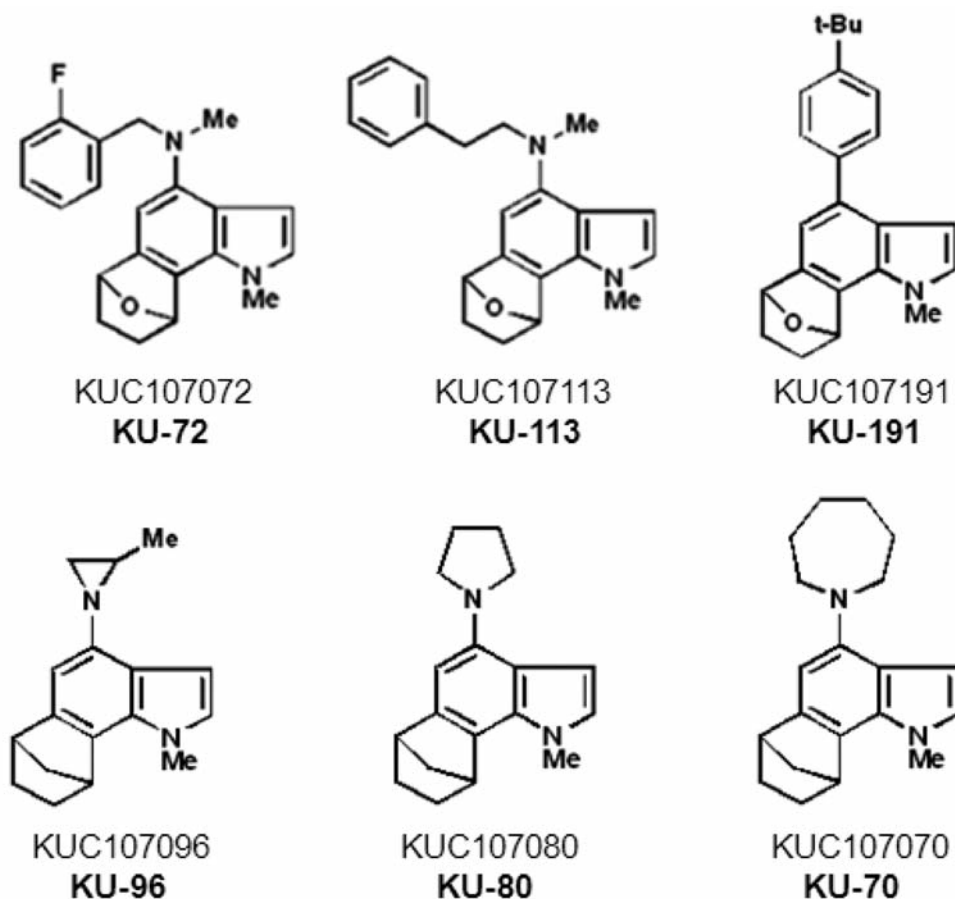


Figure 1. Chemical structures and identification numbers of the synthetic 6,7-annulated-4-substituted indole compounds tested for their antitumor effects in HL-60 cells *in vitro*.

BNCs. Known MT-disrupting agents VCR and taxol were used as positive controls in these experiments. VCR blocks tubulin polymerization and MT assembly, whereas taxol lowers the CC of free tubulin required to promote polymerization and blocks MT disassembly. JAS, a potent inducer of actin polymerization and stabilization, that interferes with actomyosin ring function and impedes cytokinesis (21-23), was also included for the sake of comparison. Treatment with 0.05  $\mu\text{M}$  VCR blocks cell-cycle progression in the M-phase and dramatically increased the percentage of mitotic cells by 9.8- and 3.7-fold at 24 and 48 h, respectively (Figure 3A). In contrast, 0.05  $\mu\text{M}$  taxol and 0.32  $\mu\text{M}$  JAS did not increase the mitotic index of HL-60 cells. Under similar conditions, 10  $\mu\text{M}$  KU-70, KU-72 and KU-80 mimic to a lesser degree the ability of VCR to block mitosis, producing 1.5-, 4.8- and 2.6-fold increases, respectively, in mitotic cells at 24 h (Figure 3A). This would suggest some MT disruption but the effects of KU-70, KU-72 and KU-80 declined or even disappeared at 48 h (Figure

3A). VCR and taxol increase BNCs by 2.6- and 5.7-fold, respectively, at 24 h but not at 48 h (Figure 3B). Based on its ability to stabilize the contractile actomyosin ring and block the cleavage furrow, the actin binder JAS (21-23) induced a 9.5-fold increase in the percentage of BNCs at 24 h that fully persists at 48 h (Figure 3B). Interestingly, KU-70, KU-72 and KU-80 treatments also produced 5.2-, 4.9- and 4.6-fold increases, respectively, in the percentage of BNCs at 24 h, these stimulatory effects declined but remained significant at 48 h (Figure 3B), indicating that the annulated indoles rapidly induce molecular events that block the process of cytokinesis in proliferating tumor cells. Control populations of vehicle-treated HL-60 tumor cells contained only 0.54% of MNCs and 0.04% of cells with MNi, indicating that these abnormal figures of mitotic disruption and genomic instability are extremely rare. The same VCR, taxol, KU-70, KU-72, KU-80 and JAS treatments shown to induce the development of BNCs also increased by 5.4-, 13.5-, 10.6-, 9.6-, 9.7- and 4.1-fold, respectively, the percentage of

Table I. Antiproliferative activity of synthetic 6,7-annulated-4-substituted indole compounds in human HL-60 tumor cells *in vitro*. Concentrations of novel synthetic indole-based compounds required to inhibit by 50% ( $IC_{50}$ ) the metabolic activity of HL-60 leukemia cells, using the MTS:PMS assay after two or four days of culture *in vitro*. The antiproliferative activity of jasplakinolide (JAS) is indicated for the sake of comparison.  $IC_{50}$  values ( $\mu M$ ) were calculated from linear regression of the slopes of the log-transformed concentration-survival curves. Data are means $\pm$ SD ( $n=3$ ). NA: Values are not available because the compound reduced tumor cell proliferation by less than 50% at the highest concentration tested (156.25  $\mu M$ ).

Compounds	$IC_{50}$ values ( $\mu M$ ) in HL-60 cells	
	Day 2	Day 4
KU-70	23.9 $\pm$ 0.6	10.3 $\pm$ 0.3
KU-72	33.8 $\pm$ 0.8	12.7 $\pm$ 0.6
KU-80	19.8 $\pm$ 0.7	12.6 $\pm$ 0.4
KU-96	33.1 $\pm$ 1.2	21.5 $\pm$ 0.6
KU-113	71.0 $\pm$ 1.6	42.4 $\pm$ 1.1
KU-191	NA	NA
JAS	0.100 $\pm$ 0.002	0.0177 $\pm$ 0.0004

MNCs at 24 h, with the effects of the annulated indoles persisting at a reduced level at 48 h (Figure 4A). This is consistent with the fact that drug-treated cells with disrupted tubulin and actin dynamics and dysfunctional mitotic spindle and cleavage furrow escape from mitosis without cytokinesis and proceed as BNCs to the next cell cycle and round of DNA synthesis to form MNCs and polyploid cells with 3-4 nuclei, which eventually die (15, 24). The VCR, taxol, KU-70, KU-72, KU-80 and JAS treatments under study also induced the development of 0.20, 0.44, 0.67, 0.57, 0.62 and 1.02%, respectively, of HL-60 cells with MNi at 24 h, with the effects of the annulated indoles remaining almost unaltered at 48 h (Figure 4B). These annulated indoles might, therefore, enhance mitotic abnormalities, induce chromosomal damage or missegregation, and block cytokinesis. Finally, as compared to 15,000 control and VCR- or taxol-treated HL-60 cells where no NPBs and nuclear buds (NBUDs) were detected, 15,000 HL-60 cells treated with KU-70, KU-72, KU-80 and JAS contained 3, 2, 6 and 2 NPBs and 5, 13, 3 and 5 NBUDs, respectively, at 48 h (data not shown). Control populations of HL-60 tumor cells incubated for 48 h in the absence of drugs contained only 0.13% of cells with five or more small nuclear structures, which is microscopic evidence of nuclear fragmentation into smaller apoptotic chromatin bodies within an intact cytoplasm and cytoplasmic membrane (15). Using such criteria, VCR had no effect but taxol, KU-70, KU-72, KU-80 and JAS treatments increased the percentage of microscopically-visible apoptotic tumor cells by 86.2-, 9.8-, 5.0-, 2.7- and 20.1-fold, respectively (data not shown).

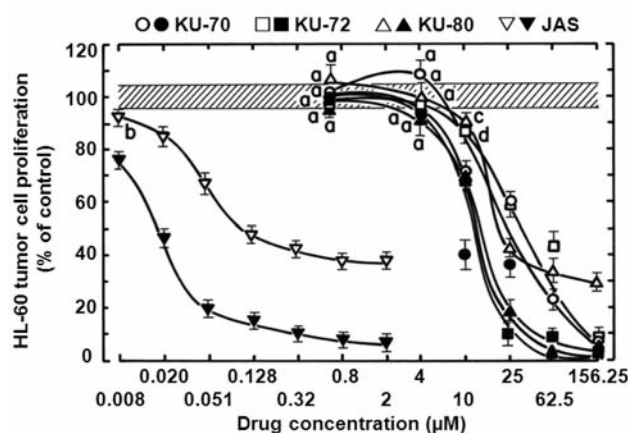


Figure 2. Comparison of the ability of serial concentrations (logarithmic intervals) of KU-70, KU-72 and KU-80 to inhibit the metabolic activity of HL-60 tumor cells at days 2 (open symbols) and 4 (solid symbols) *in vitro*. The antiproliferative activities of jasplakinolide (JAS) concentrations at days 2 and 4 are indicated for the sake of comparison. HL-60 cell proliferation results are expressed as percentage of the net absorbance of MTS/formazan after bioreduction by vehicle-treated control cells after two ( $A_{490\text{ nm}}=1.179\pm 0.048$ ) and four ( $A_{490\text{ nm}}=1.149\pm 0.047$ ) days in culture ( $100\pm 4.1\%$ , striped area). The blank values ( $A_{490\text{ nm}}=0.370$  at day 2 and  $0.439$  at day 4) for cell-free culture medium supplemented with MTS:PMS reagent were subtracted from the results. Data are means $\pm$ SD ( $n=3$ ). <sup>a</sup>Not different from respective controls; <sup>b</sup> $p<0.05$ , <sup>c</sup> $p<0.025$  and <sup>d</sup> $p<0.01$ , significantly lower than respective controls.

**Inhibition of tubulin polymerization.** The classic turbidity assay was used to study the effects of anti-proliferative annulated indoles on tubulin polymerization in the presence or absence of glycerol. Normally, glycerol and taxol stabilize tubulin and lower the CC of protein required to initiate MT assembly (16). As shown by the control curve a concentration of purified tubulin below 10 mg/ml cannot polymerize in the absence of 15% glycerol (Figure 5A). However, the MT-stabilizing drug taxol can easily induce the polymerization of such a low concentration of tubulin (1.15 mg/ml) in the absence of 15% glycerol (Figure 5A). In contrast to 10  $\mu M$  taxol, 300  $\mu M$  KU-72 were not able to promote tubulin polymerization in the absence of glycerol and, thus, is KU-72 not a MT-stabilizing agent that blocks MT disassembly like taxol (Figure 5A). The control curve in Figure 5B shows the three typical phases of MT assembly taking place when purified tubulin (2.25 mg/ml) undergoes polymerization in the presence of 15% glycerol: a short lag phase, an exponential growth phase almost linear between 100 and 400 s, and a steady phase reaching a plateau after 520 s (16). At 10  $\mu M$ , compared to the control, VCR almost totally inhibited the rate and extent of glycerol-induced tubulin polymerization by 94.5% and 93.7%, respectively (Figure 5B). At 300  $\mu M$ , KU-72 mimics the inhibitory effect of VCR and reduces the rate and extent of glycerol-induced tubulin polymerization by 72.2% and

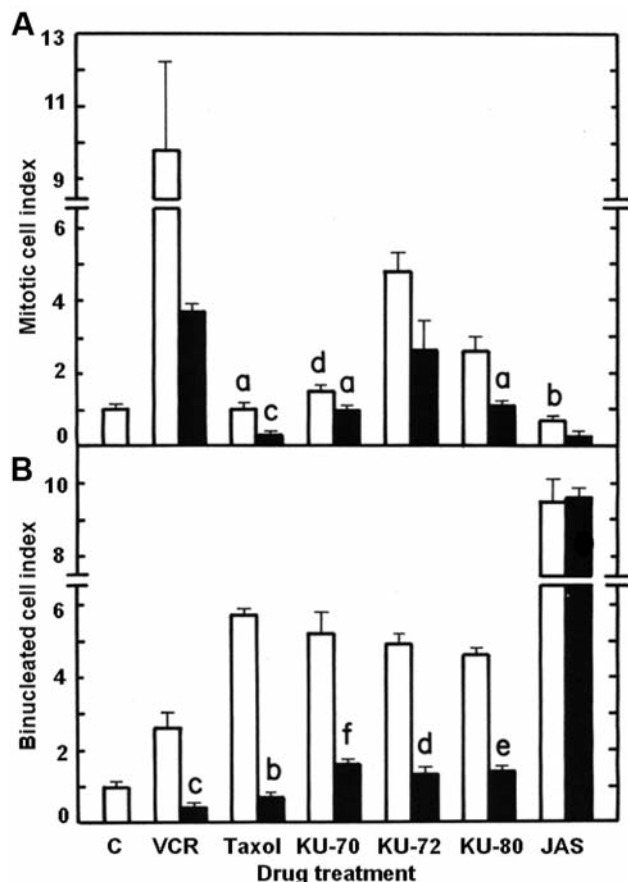


Figure 3. Comparison of the effects of KU-70 (10  $\mu$ M), KU-72 (10  $\mu$ M), KU-80 (10  $\mu$ M), vincristine (VCR, 0.05  $\mu$ M), taxol (0.05  $\mu$ M) and jasplakinolide (JAS, 0.32  $\mu$ M) on the frequency of mitotic figures (A) and bi-nucleated cells (B) in HL-60 tumor cells in vitro. HL-60 cells were incubated in triplicate for 24 (open columns) and 48 h (closed columns) at 37°C in the presence or absence (C: control) of the indicated drugs. The percentage of cells in each category was determined by morphological analysis, scoring an average of approximately 5,000 cells/slide to identify those containing mitotic figures or two nuclei. Results are expressed as the percentage of mitotic (A) or bi-nucleated cells (B) in drug-treated cultures relative to the mitotic (C: 1.34 $\pm$ 0.12%) or binucleated (C: 2.66 $\pm$ 0.23%) cells in vehicle-treated controls. Data are means $\pm$ SD (n=3). <sup>a</sup>Not different from control; <sup>b</sup>p<0.005 and <sup>c</sup>p<0.0005, significantly smaller than respective controls in A and B; <sup>d</sup>p<0.025, <sup>e</sup>p<0.005 and <sup>f</sup>p<0.0005, significantly greater than respective controls in A and B.

70.5%, respectively, suggesting that this annulated indole, which increased the mitotic cell index like VCR (Figure 3A), might also be a MT de-stabilizing drug that prevents MT assembly (Figure 5B). Glycerol-induced tubulin polymerization between 80 and 520 s is inhibited by 93.7% in the presence of 10  $\mu$ M of the known tubulin binder VCR but remains unaltered in the presence of 5  $\mu$ M of the known actin binder JAS (Figure 6). In contrast, the three annulated indoles, which increased the

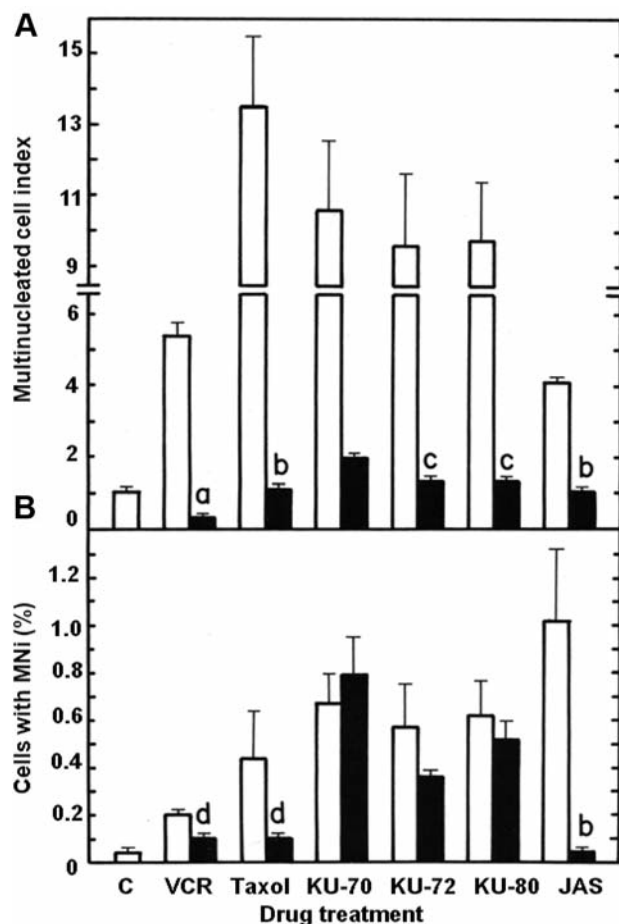


Figure 4. Comparison of the effects of KU-70 (10  $\mu$ M), KU-72 (10  $\mu$ M), KU-80 (10  $\mu$ M), vincristine (VCR, 0.05  $\mu$ M), taxol (0.05  $\mu$ M) and jasplakinolide (JAS, 0.32  $\mu$ M) on the frequency of multi-nucleated cells (A) and micronuclei (B) in HL-60 tumor cells in vitro. HL-60 cells were incubated in triplicate for 24 (open columns) and 48 h (closed columns) at 37°C in the presence or absence (C: control) of the indicated drugs. The percentage of cells in each category was determined by morphological analysis, scoring an average of about 5,000 cells/slide to identify those containing 3-4 nuclei or MNI. A: Results are expressed as the percentage of multi-nucleated cells in drug-treated cultures relative to the multi-nucleated cells (C: 0.54 $\pm$ 0.04%) in vehicle-treated controls. B: Results are expressed as the percentage of vehicle- (C: 0.04 $\pm$ 0.002%) or drug-treated cells with MNI. Data are means $\pm$ SD (n=3). <sup>a</sup>p<0.0005, significantly smaller than control; <sup>b</sup>Not different from respective controls in A and B; <sup>c</sup>p<0.025, and <sup>d</sup>p<0.0005, significantly greater than respective controls in A and B.

mitotic index of HL-60 tumor cells, are also capable of inhibiting the polymerization of tubulin in the cell-free turbidity assay. Indeed, 120  $\mu$ M KU-70 and 300  $\mu$ M KU-80 reduced the rate of glycerol-induced tubulin polymerization by 57.9% and 59.0%, respectively, whereas 48, 120 and 300  $\mu$ M KU-72 clearly induced concentration-dependent inhibitory effects that reduced tubulin polymerization by 23.0, 46.3 and 70.5%, respectively (Figure 6).

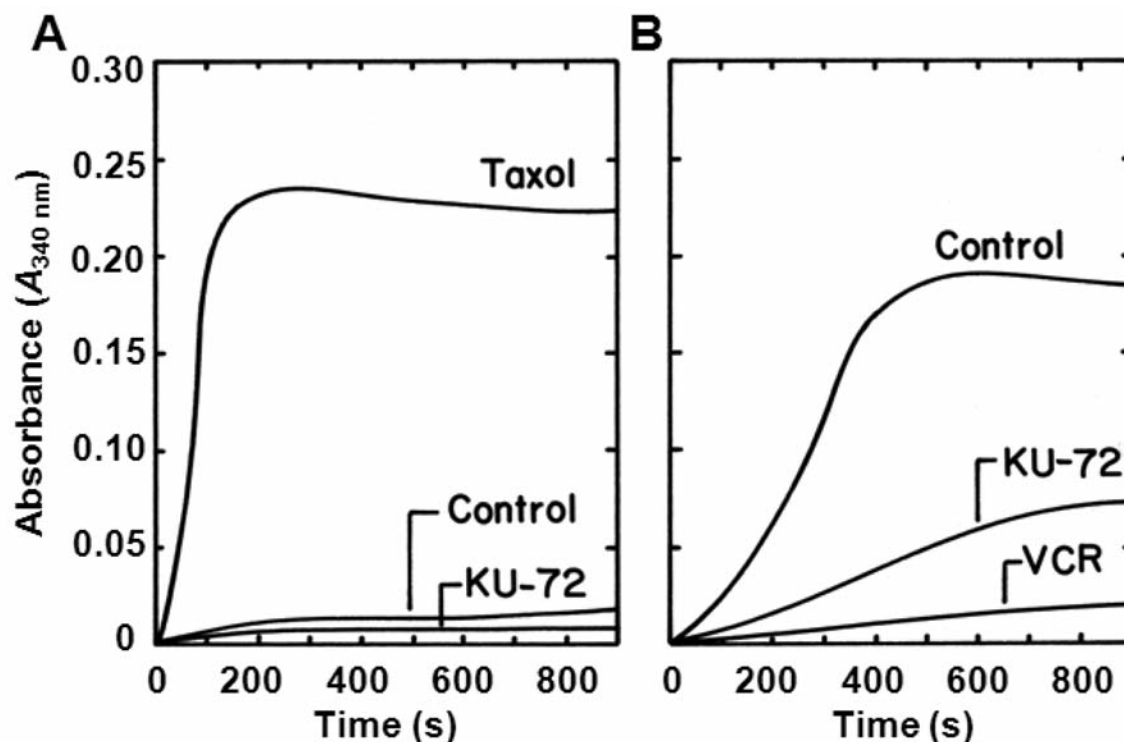


Figure 5. Comparison of the ability of antiproliferative KU-72 and known microtubule (MT)-stabilizing (A) and MT de-stabilizing (B) anticancer drugs to respectively alter the kinetics of tubulin polymerization in the absence (A) or presence (B) of glycerol *in vitro*. A: Purified tubulin was diluted to a final concentration of 1.15 mg/ml in 80 mM PIPES buffer, pH 6.9, containing 10 mM MgCl<sub>2</sub>, 1 mM EGTA and 1 mM GTP. The polymerization reactions were placed in quartz microcells and incubated at 35°C in the presence or absence (control) of 300 μM KU-72 or 10 μM taxol. B: The turbidity assay mixtures were identical to those of (A) but contained 2.25 mg tubulin/ml and 15% glycerol. The polymerization reactions were similarly incubated in the presence or absence (control) of 300 μM KU-72 or 10 μM vincristine (VCR). The rate of MT assembly was continuously monitored by scanning over 900 s the increase in turbidity (absorbance at 340 nm). Assays were performed in duplicate.

**Stimulation of actin polymerization.** As indicated by the control curve in Figure 7, the nucleation and polymerization of purified G-actin (0.45 mg/ml) to form F-actin can be induced by an actin-polymerizing buffer containing Mg<sup>2+</sup> and ATP and quantified by the fluorescence intensity of N-(1-pyrenyl)iodoacetamide that increases as it becomes incorporated into the double-helical structure of F-actin filaments (19, 20). In relation to its ability to induce actin nucleation, polymerization and stabilization (21-23), above 0.256 μM, JAS accelerates, in a concentration-dependent manner, the rate at which the fluorescence of pyrene-labeled actin develops to reach a plateau of maximal F-actin polymerization (Figure 7). Interestingly, most of the antiproliferative annulated indoles, except KU-96 which has a marginal effect, appear to increase both the early rate and maximal plateau of pyrene-labeled actin polymerization (Figure 8) but the slower acceleration and greater magnitude of these responses, compared with the control, suggests that their interaction with actin might be slightly different from that of JAS (Figure 7). Indeed, 250 μM KU-96, KU-191, KU-80, KU-113, KU-72 and KU-70 respectively increased the rate of

actin polymerization by 1.2-, 1.5-, 1.6-, 1.6-, 1.8- and 2.0-fold compared to control at 4 min. After all curves reach a plateau at 19 min, these differences in the maximal levels of F-actin formed persisted up to 30 min (Figure 8). Using the most effective annulated indole shown in Figure 8, the concentration-dependent ability of KU-70 to stimulate pyrene-labeled actin polymerization in this fluorimetric assay is demonstrated and substantiates the observation that annulated indoles increase both the early rate and maximal level of F-actin formation (Figure 9). KU-70 at 25, 62.5, 156.25 and 390.625 μM increased the rate of actin polymerization by 1.3-, 1.4-, 1.7- and 3.1-fold, respectively, compared with the control at 4 min and, when the curves reached a plateau at about 9 min, these differences in the maximal level of F-actin formed persisted up to 25 min (Figure 9).

## Discussion

Since annulated indole structures are virtually unknown, it is important to demonstrate that some members of the first polycyclic annulated indole library constructed using a

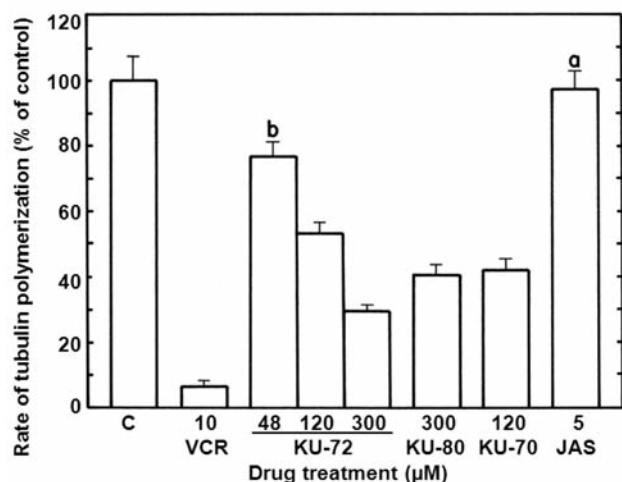


Figure 6. Comparison of the ability of anti-proliferative KU-72, KU-80 and KU-70, jasplakinolide (JAS) and the known microtubule destabilizing anticancer drug vincristine (VCR) to alter the kinetics of glycerol-induced tubulin polymerization *in vitro*. The conditions of the assays were identical to those of Figure 5B. The polymerization reactions were incubated in the presence or absence (control) of 10 μM VCR, 48, 120 or 300 μM KU-72, 300 μM KU-80, 120 μM KU-70, and 5 μM JAS. Results are expressed as the percentage of the rate of glycerol-induced tubulin polymerization based on the linear increase in turbidity between 80 and 520 s in vehicle-treated control assays ( $\Delta_{A340_{nm}}=0.190\pm0.012$ ;  $100\pm6.6\%$ ). Data are means $\pm$ SD (n=2). <sup>a</sup>Not different from control; <sup>b</sup>p<0.05, significantly less than control.

tandem indole aryne cycloaddition/cross-coupling sequence of reactions with the 4,6,7-tribromoindole platform (1-7, 12, 25) can induce antitumor effects in the micromolar range in both the L1210 (13) and HL-60 cell systems. Interestingly, the five annulated indoles that most inhibited HL-60 tumor cell proliferation were from the Buchwald-Hartwig cross-coupled products (*i.e.* those with an amine at the C-4 position of the indole), whereas the weaker antiproliferative KU-191 compound came from the Suzuki-Miyaura series of library members (13). The concentration-dependent inhibition of HL-60 tumor cell proliferation by the annulated indoles is always more pronounced at four rather than two days, suggesting that the efficacy of these bioactive compounds is a combination of their concentration and duration of action. Because annulated indoles increasingly block HL-60 cells in mitosis and slow down their proliferation rate, the difference between the number of exponentially growing untreated control cells and the number of drug-treated cells continues to increase over time in culture. KU-70, KU-72, KU-80, KU-96, KU-113 and KU-191 were selected for the present HL-60 study because they had the best anti-proliferative IC<sub>50</sub> values in L1210 cells at day 2 in relation with their ability to inhibit DNA synthesis at 3 h and induce DNA fragmentation at 24 h. This suggests

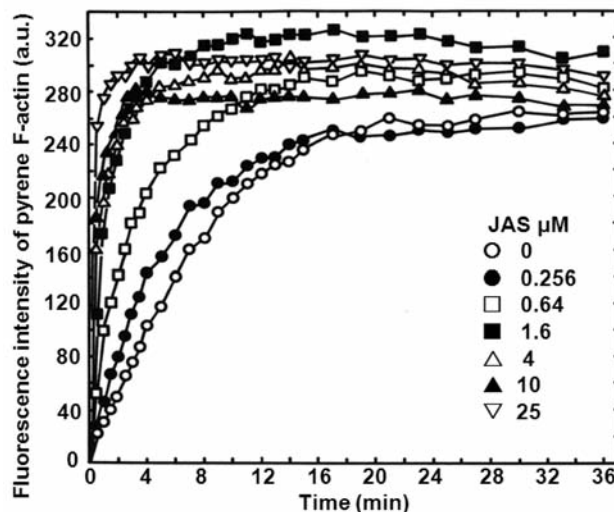


Figure 7. Comparison of the ability of serial concentrations of jasplakinolide (JAS) to alter the kinetics of actin polymerization *in vitro*. Assay mixtures (200 μl) containing pyrene G-actin (0.45 mg/ml) in 10 mM Tris-HCl buffer, pH 7.5, with 0.2 mM CaCl<sub>2</sub>, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 5 mM guanidine carbonate and 1 mM ATP, were placed in 96-well opaque assay plates and polymerization reactions were initiated at room temperature in the absence or presence of JAS. The increasing fluorescence of pyrene F-actin was monitored every 30 s to 3 min over a 36-min period at 365 nm excitation and 407 nm emission. The baseline fluorescence of pyrene-labeled G-actin ( $36.2\pm7.8$  arbitrary units) in the absence of polymerization-inducing buffer was subtracted from the results. Data points are the means of replicates from three different experiments.

that these annulated indoles are the most effective in short-term antitumor assays and might have a more rapid mechanism of action, needing less time to interact with and be incorporated into cells, undergoing eventual metabolic activation to reach crucial cellular targets, and trigger various inhibitory pathways that finally damage and kill tumor cells (13). The fact that the IC<sub>50</sub> values of these compounds required to inhibit the mitochondrial ability of tumor cells to metabolize MTS:PMS reagent at day 4 are slightly higher in HL-60 (10.3-42.4 μM) than L1210 cells (2.0-4.0 μM) (13) might simply be due to the different rates of growth the cell lines. Since suspension cultures of 10<sup>5</sup> viable L1210 and HL-60 leukemia cells multiply 21- to 24-fold and 6- to 7-fold, respectively, within seven days when fresh medium is replenished, the anti-proliferative action of annulated indoles might be more effective against unsynchronized populations of rapidly-growing tumor cells that are frequently turning through the cell cycle than against unsynchronized populations of relatively slow-growing tumor cells that have smaller growth fractions and divide less frequently.

Tubulin is a labile protein, unstable below 80 mM PIPES, and should not be exposed to pH<6.8 or pH>7.0 and will not polymerize in the presence of Ca<sup>2+</sup> (16). The propensity of tubulin subunits to assemble into MTs is dependent upon their



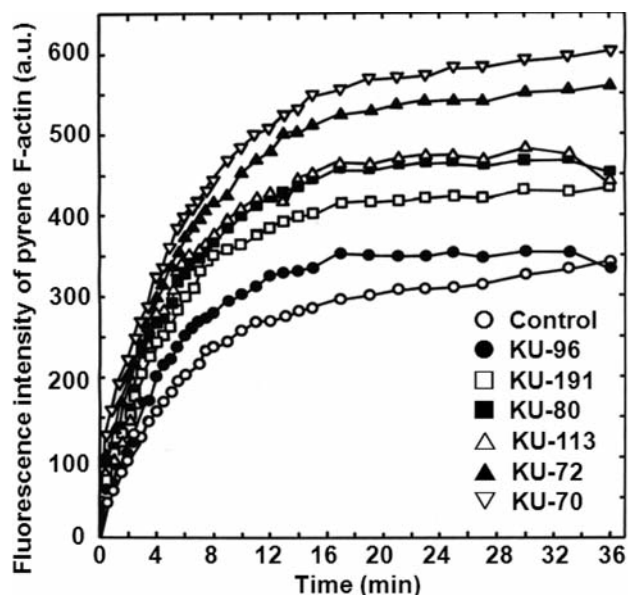


Figure 8. Comparison of the ability of various anti-proliferative KU compounds to alter the kinetics of actin polymerization *in vitro*. Assay mixtures (200  $\mu$ l) containing pyrene G-actin (0.45 mg/ml) in 10 mM Tris-HCl buffer, pH 7.5, with 0.2 mM  $\text{CaCl}_2$ , 50 mM KCl, 2 mM  $\text{MgCl}_2$ , 5 mM guanidine carbonate and 1 mM ATP, were placed in 96-well opaque assay plates and polymerization reactions were initiated at room temperature in the absence (control) or presence of 250  $\mu$ M KU-96, KU-191, KU-80, KU-113, KU-72 and KU-70. The increasing fluorescence of pyrene F-actin was monitored every 30 s to 3 min over a 36-min period at 365 nm excitation and 407 nm emission. The baseline fluorescence of pyrene-labeled G-actin ( $38.5 \pm 8.6$  arbitrary units) in the absence of polymerization-inducing buffer was subtracted from the results. Data points are the means of replicates from three different experiments.

affinity for MT ends. In order to achieve MT assembly, this affinity (the CC) has to be less than the total concentration of free tubulin subunits (16). GTP and  $\text{Mg}^{2+}$  are necessary for tubulin nativity, and glycerol stabilizes tubulin and lowers the CC required to initiate polymerization (16). Taxol, which also lowers the CC and eliminates the requirement for GTP, promotes tubulin polymerization in the absence of glycerol and stabilizes MTs by inhibiting their depolymerization (26, 27). The effect of taxol on tubulin without glycerol and the effect of glycerol on tubulin, therefore, can be used to screen for MT-stabilizing and de-stabilizing drugs (26). A short lag phase is necessary to create nucleation sites, which are small tubulin oligomers from which larger MT polymers can form. Because MT polymerization is readily reversible, a given population of MTs is continually growing and shortening, a phenomenon called dynamic instability (16). Thus, the growth phases observed in Figure 5 reflect the rapid increase in the ratio of MT assembly to disassembly. Finally, a steady phase is established when the residual concentration of free tubulin heterodimer equals the CC required to initiate polymerization

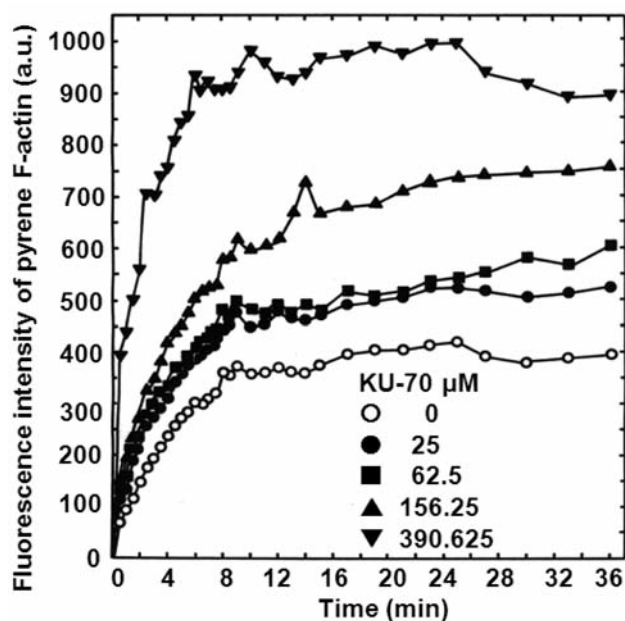


Figure 9. Comparison of the ability of serial concentrations of anti-proliferative KU-70 to alter the kinetics of actin polymerization *in vitro*. Assay mixtures (200  $\mu$ l) containing pyrene G-actin (0.45 mg/ml) in 10 mM Tris-HCl buffer, pH 7.5, with 0.2 mM  $\text{CaCl}_2$ , 50 mM KCl, 2 mM  $\text{MgCl}_2$ , 5 mM guanidine carbonate and 1 mM ATP, were placed in 96-well opaque assay plates and polymerization reactions were initiated at room temperature in the absence or presence of KU-70. The increasing fluorescence of pyrene F-actin was monitored every 30 s to 3 min over a 36-min period at 365 nm excitation and 407 nm emission. The baseline fluorescence of pyrene-labeled G-actin ( $40.2 \pm 9.4$  arbitrary units) in the absence of polymerization-inducing buffer was subtracted from the results. Data points are the means of replicates from three different experiments.

(16). The rate and extent of taxol-induced tubulin polymerization may be faster and higher than those induced by glycerol because taxol blocks MT disassembly. The kinetics of taxol-, and glycerol-induced MT polymerization shown in Figure 5 appear consistent with the initial concentrations of 1.15-2.25 mg tubulin/ml used in our reactions.

In contrast to the MT-stabilizing drug taxol (26, 27), a full antiproliferative concentration of KU-72 cannot induce the polymerization of tubulin without glycerol, suggesting that this annulated indole neither promotes MT assembly nor blocks tubulin de-polarization and MT disassembly. However, KU-72 inhibits the rate and extent of glycerol/ $\text{Mg}^{2+}$ -induced polymerization of purified tubulin in a concentration-dependent manner, demonstrating that this annulated indole blocks MT assembly similarly to the known MT de-stabilizing drugs that bind to tubulin on the colchicine or vinblastine sites (26). KU-72 might inhibit tubulin polymerization as a consequence of its binding interaction with the  $\alpha/\beta$  tubulin dimer, although other mechanisms, such as interactions with  $\text{Mg}^{2+}$  ions or GTP, are

conceivable (26). The *Vinca* alkaloid VCR is a spindle poison which binds to tubulin, preventing MT assembly, causing metaphase arrest and thus killing cells attempting mitosis (26, 28). Although less strongly than VCR, annulated indoles can inhibit the polymerization of purified tubulin in a cell-free system but at concentrations much higher than those shown to increase the mitotic index and inhibit the proliferation of L1210 (13) and HL-60 cells. The fact that the concentrations of anti-mitotic agents effective in the tubulin polymerization assay are consistently higher than those with cytostatic activity has been noticed before (17, 18, 26, 29). Anti-mitotic drugs interacting with a few essential sites in the MTs might disrupt the mitotic spindle and be cytostatic over a 2- to 4-day period at concentrations much lower than those required to directly block the rate of glycerol/Mg<sup>2+</sup>-induced tubulin polymerization in a cell-free turbidity assay (17, 18, 26, 29). Indeed, mitotic arrest occurs when less than 5% of the cellular tubulin is complexed by colchicine (26). Nevertheless, the ability of annulated indoles to inhibit tubulin polymerization and increase the mitotic index suggests that these anti-tubulin and anti-mitotic effects might play a role, at least in part, in the mechanism of their anti-proliferative and anti-tumor activities.

Based on their ability to inhibit tubulin polymerization and disrupt MT dynamics, KU-70, KU-72 and KU-80 would be expected to arrest cells in the G<sub>2</sub>/M phase. The mitotic index can differentiate between the anti-mitotic drugs that cause G<sub>2</sub> or M phase arrest. Agents that arrest cells in the M phase, such as VCR, increase the mitotic index but agents that cause G<sub>2</sub> arrest, such as etoposide (VP-16), reduce it. Since KU-70, KU-72 and KU-80 increase the percentage of mitotic figures and the mitotic index within 24 h, these annulated indoles might be capable of causing metaphase arrest and blocking the progression of HL-60 tumor cells in the M phase of their cycle, although to a lesser degree than VCR. When MT assembly is prevented in colchicine- or nocodazole-treated cells, the level of unpolymerized tubulin is increased and this, in turn, inhibits the formation of new tubulin mRNA while the pre-existing message decays rapidly (30). Since annulated indoles also inhibit DNA synthesis in L1210 cells (13), the ability of these anti-proliferative compounds to affect the level of translatable tubulin mRNA, decrease tubulin production and alter other phases of the cell cycle should be studied.

Anti-proliferative concentrations of annulated indoles induce a time-dependent increase of caspase-3 activity, which peaks at 6 h in L1210 cells and precedes the fragmentation of radiolabeled DNA and the appearance of apoptotic cells with five or more small nuclear structures at 24 h, suggesting that the ability of annulated indoles to trigger apoptosis might play a significant role in their molecular mechanism of antitumor activity (13). Even though most of the anti-proliferative indoles, except KU-80,

do not directly bind to purified DNA to inhibit the fluorescence of the ethidium bromide-DNA complex (13), the abilities of these anti-tumor compounds to interact with maintenance enzymes or regulatory proteins and indirectly cause high molecular weight DNA-strand breaks, crosslinks or chromosome aberrations and cytokinesis disruption should be determined. Besides some tubulin interaction to disrupt the polymerization of MTs, annulated indoles are likely to target other molecular events in order to elicit their anti-proliferative activity. For instance, anti-proliferative concentrations of KU-69, KU-70, KU-72 and KU-80 might trigger genotoxic effects that induce chromosomal aberration, disrupt chromosomal segregation and block cytokinesis to substantially increase the levels of BNCs, MNCs and MNi in both L1210 (13) and HL-60 cells at 24-48 h, the magnitudes of these responses often matching or even surpassing those induced by VCR and taxol.

The presence of BNCs indicate that cytokinesis is inhibited following nuclear division (15). MNCs may follow if BNCs re-enter the cell cycle and escape further cytokinesis. The MNi test is an indicator of drug-induced chromosomal damage and aberration as such MNi structures generally form during the metaphase/anaphase transition of mitosis when a whole lagging chromosome or an acentric chromosome fragment resulting from a clastogenic or mutagenic event fail to integrate into the daughter nuclei (15). However, chromosome aberration and non-disjunction/missegregation might be the consequence of prolonged drug-induced mitotic disruption and might be responsible for the inability to undergo cytokinesis after regression of the cleavage furrow (31). If completion of cytokinesis requires accurate chromosome segregation, annulated indoles might induce chromosome aberration and missegregation to increase the frequency of BNCs, MNCs and MNi in L1210 (13) and HL-60 cells. NPBs between nuclei in BNCs originate from dicentric chromosomes in which the centromeres have been pulled to the opposite poles of the cell at anaphase and are indicative of DNA mis-repair, chromosome re-arrangement or telomere end-fusions (15). NPBs may break to form MNi. NPBs, which are correlated with MNi frequency in BNCs, are thus biomarkers of dicentric chromosomes and might provide indirect evidence of annulated indole-induced genome damage resulting from misrepaired DNA breaks. NBUDs occur during the S-phase and form MNi that are still linked to the nucleus by a narrow or wide stalk of nucleoplasmic material, depending on the stage of the budding process. Amplified or excess DNA is selectively localized to specific sites at the periphery of the nucleus and is eliminated *via* nuclear budding to form MNi during the S phase. Hence, annulated indoles and drugs that inhibit DNA synthesis such as hydroxyurea might increase the rate of elimination of amplified DNA and possibly DNA-repair complexes *via* nuclear budding (32, 33).

An interesting finding is that these annulated indoles mimic the ability of JAS to stimulate the rate and level of F-actin polymerization, an event which could stabilize the cleavage furrow, inhibit cytokinesis and account for the formation of many BNCs after KU-70, KU-72, KU-80 and JAS treatments. Cytokinesis is a complex and multistep process of temporarily and spatially coordinated action of the cell cycle and cytoskeletal and membrane systems to achieve physical separation of daughter cells by constriction of a cleavage furrow. Because major cytokinesis proteins include components of the cleavage furrow, such as actin, myosin and anillin, as well as signaling systems that position and regulate the furrow, such as aurora B and Polo kinases and Rho family GTPases, most compounds currently known to inhibit cytokinesis are natural-product actin binders and small-molecule inhibitors of aurora B kinase. Both newly-polymerized and pre-existing F-actin filaments can contribute to the initial assembly of the contractile ring of actin and myosin that cleaves the cell into two. Once formed, the ring remains a dynamic structure in which actin and other ring components continuously assemble and disassemble (34). Contractile ring formation requires highly regulated and localized F-actin assembly, whereas its constriction depends upon the action of myosin (35). Once the dense, bundled F-actin network of the contractile ring is formed, myosin pulls on the actin fibers to invaginate the plasma membrane. Soon after, components of the contractile ring must be removed or degraded to allow disassembly of the ring. JAS, a macrocyclic peptide isolated from the sponge *Jaspis johnstoni*, competes with phalloidin for actin binding and is a potent inducer of F-actin nucleation, polymerization and stabilization (21, 22). Because JAS is cell permeant, exhibits fungicidal, insecticidal and anti-proliferative activities, and enhances apoptosis (36-39), this peptide is useful in studying the role of actin dynamics in cell processes such as adhesion, locomotion, endocytosis, vesicle sorting and release, and cytokinesis. Our results are consistent with the reports that an excessive accumulation and delocalization of F-actin caused by JAS is linked to cytokinesis failure, multinucleation, MNI and apoptosis (21-23).

Since actin dynamics are required to assemble and disassemble the contractile actomyosin ring that cleaves the mother cell into two daughter cells, actin binders, such as JAS, that stimulate aberrant actin nucleation, polymerization, stabilization and mislocalization might prevent constriction of the cleavage furrow after nuclear division, block cytokinesis and induce bi- and multi-nucleation, resulting to genomic instability and apoptosis. Excessive polymerization and stabilization of F-actin that interferes with the functionality of the actomyosin ring during cytokinesis, therefore, might also play a role in the mechanism by which annulated indoles inhibit cytokinesis to cause multinucleation.

This hypothesis is substantiated by several studies demonstrating that stabilized F-actin structures cause massive cell division defects. For instance, cofilin, a

regulator of actin cytoskeletal dynamics essential for F-actin turnover, is inhibited by phosphorylating kinases and activated by dephosphorylating phosphatases, such as chronofin. Hence, chronofin-expressing cells with active cofilin can depolymerize F-actin to avoid excessive F-actin accumulation, whereas chronofin-depleted cells with increased levels of inactive phosphocofilin have aberrant accumulation of F-actin, persistent contractile rings, and cytokinesis failure, resulting in the formation of BNCs and MNCs (40). Another example is that specific mutations of the Wiskott-Aldrich syndrome protein (WASp) that compromise normal auto-inhibition of WASp result in unregulated activation of the actin-related protein 2/3 (ARP2/3) complex, which is essential for WASp-mediated actin nucleation, and enhanced and delocalized F-actin polymerization that cause defects of mitosis and cytokinesis in X-linked neutropenia (23, 41). Transgenic expression of mutant WASp caused hyperactivation and mislocalization of F-actin polymerization, which led to the formation of BNCs, MNCs and MNI, indicative of aborted cytokinesis and genomic instability (23, 41). The increased and delocalized F-actin induced by autoactive WASp during interphase persists during all stages of mitosis, surrounding condensed chromosomes and the mitotic spindle, and accumulating around the spindle midzone during furrow ingression (23, 41). Conversely, prevention of excess F-actin production in cells expressing mutant WASp reverses all the antiproliferative and nuclear effects and also partially restores normal mitotic timing (23, 42). The key features of X-linked neutropenia, therefore, can all be traced to a nonspecific mechanical disruption of cell division caused by an excess of cytoplasmic F-actin polymerization (23, 41, 42).

Direct physical inhibition is speculated to explain how excess F-actin, causing increased cellular viscosity, might slow down all phases of mitosis, increase the frequency of lagging chromosomes and block cytokinesis (23, 42). F-Actin accumulation around the mitotic chromosomes might form a physical barrier inhibiting chromosome capture and separation by spindle MTs. A persistent F-actin network traversing the cytoplasmic bridge left after actomyosin ring closure might similarly impede cytokinesis by preventing abscission of the daughter cells. Alternatively, excessive F-actin surrounding the spindle midzone during late anaphase might inhibit the signal from the spindle midzone to the cell cortex that is essential for furrowing and cytokinesis (23, 42). Similarly, formins contribute to the elongation of F-actin filaments to form the dynamic contractile actomyosin ring but must be switched off or degraded to allow disassembly of the contractile ring during cytokinesis (43). Forced expression of activated formins, therefore, is linked to hyperactivation and mislocalization of F-actin polymerization, suggested to impede the intracellular trafficking of membrane to the central spindle so crucial for cleavage furrow invagination and subsequent separation (43).

Overall, synthetic 6,7-annulated 4-substituted indoles might be multi-functional anti-proliferative compounds advantageous for disrupting both tubulin and actin dynamics, thereby inducing mitotic abnormalities, cytokinesis failure, genomic instability and apoptosis. Further structure–activity relationships are required to identify more potent antitumor lead compounds and characterize additional molecular targets and mechanisms of action.

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### References

- Buszek KR, Luo D, Kondrashov M, Brown N and VanderVelde D: Indole-derived arynes and their Diels-Alder reactivity with furans. *Org Lett* 9: 4135-4137, 2007.
- Buszek KR, Brown N and Luo D: Concise total synthesis of (±)-cis-trikentrin A and (±)-herbindol A via intermolecular indole aryne cycloaddition. *Org Lett* 11: 201-204, 2009.
- Chandrasoma N, Brown N, Brassfield A, Nerurkar A, Suarez S and Buszek KR: Total synthesis of (±)-cis-trikentrin B via intermolecular 6,7-indole aryne cycloaddition and Stille cross-coupling. *Tetrahedron Lett* 54: 913-917, 2013.
- Brown N, Luo D, VanderVelde D, Yang S, Brassfield A and Buszek KR: Regioselective Diels-Alder cycloadditions and other reactions of 4,5-, 5,6-, and 6,7-indole arynes. *Tetrahedron Lett* 50: 63-65, 2009.
- Brown N, Luo D, Decapo JA and Buszek KR: New synthesis of (±)-cis-trikentrin A via tandem indole aryne cycloaddition/Negishi reaction. Applications to library development. *Tetrahedron Lett* 50: 7113-7115, 2009.
- Garr AN, Luo D, Brown N, Cramer CJ, Buszek KR and VanderVelde D: Experimental and theoretical investigations into the unusual regioselectivity of 4,5-, 5,6-, and 6,7-indole aryn cycloadditions. *Org Lett* 12: 96-99, 2010.
- Nerurkar A, Chandrasoma N, Maina L, Brassfield A, Luo D, Brown N and Buszek KR: Further investigations into the regioselectivity of 6,7-indole aryne cycloadditions with 2-substituted furans: Remarkable contrasteric preference depends on pyrrole and benzene ring substitution. *Synthesis* 45: 1843-1852, 2013.
- Jackson SK and Kerr MA: Total synthesis of (±)-herbindole A, (±)-herbindole B, and (±)-cis-trikentrin A. *J Org Chem* 72: 1405-1411, 2007.
- Jackson SK, Banfield SC and Kerr MA: Total synthesis of (±)-herbindole B, and (±)-cis-trikentrin B. *Org Lett* 7: 1215-1218, 2005.
- Hitotsuyanagi Y, Fujiki H, Suganuma M, Aimi N, Sakai S-I, Endo Y, Shudo K and Sugimura T: Isolation and structure elucidation of teleocidin B-1, B-2 and B-4. *Chem Pharm Bull* 32: 4233-4236, 1984.
- Singh SB, Ondeyka JG, Jayasuriya H, Zink DL, Ha SN, Dahl-Roshak A, Greene J, Kim JA, Smith MM, Shoop W and Tkacz JS: Nodulisporic acids D-F: Structure, biological activities, and biogenetic relationships. *J Nat Prod* 67: 1496-1506, 2004.
- Thornton PD, Brown N, Hill D, Neuenswander B, Lushington GH, Santini C and Buszek KR: Application of 6,7-indole aryne cycloaddition and Pd(0)-catalyzed Suzuki-Miyaura and Buchwald-Hartwig cross-coupling reactions for the preparation of annulated indole libraries. *ACS Comb Sci* 13: 443-448, 2011.
- Perchellet J-P, Waters AM, Perchellet EM, Thornton PD, Brown N, Hill D, Neuenswander B, Lushington GH, Santini C, Chandrasoma N and Buszek KR: Antitumor effects of synthetic 6,7-annulated-4-substituted indole compounds in L1210 leukemic cells *in vitro*. *Anticancer Res* 32: 4671-4684, 2012.
- Cory AH, Owen JC, Barltrop JA and Cory JG: Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun* 3: 207-212, 1991.
- Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S and Zeiger E: HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat Res* 534: 65-75, 2003.
- Engelborghs Y: Dynamic aspects of microtubule assembly. *In: Microtubule Proteins*. Avila J (ed.). Boca Raton, FL, CRC Press, pp. 1-35, 1990.
- Perchellet EM, Ladesich JB, Chen Y, Sin H-S, Hua DH, Kraft SL and Perchellet J-P: Antitumor activity of tricyclic pyrone analogs, a new synthetic class of microtubule de-stabilizing agents, in the murine EMT-6 mammary tumor cell line *in vitro*. *Anti-Cancer Drugs* 9: 565-576, 1998.
- Perchellet EM, Ladesich JB, Collery P and Perchellet J-P: Microtubule-disrupting effects of gallium chloride *in vitro*. *Anti-Cancer Drugs* 10: 477-488, 1999.
- Kouyama T and Mihashi K: Fluorimetry study of N-(1-pyrenyl)iodoacetamide-labelled F-actin. Local structural change of actin promoter both on polymerization and on binding of heavy meromyosin. *Eur J Biochem* 114: 33-38, 1981.
- Cooper JA, Walker SB and Pollard TD: Pyrene actin: Documentation of the validity of a sensitive assay for actin polymerization. *J Muscle Res Cell Motil* 4: 253-262, 1983.
- Bubb MR, Senderowicz AMJ, Sausville EA, Duncan KLK and Korn ED: Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J Biol Chem* 269: 14869-14871, 1994.
- Bubb MR, Spector I, Beyer BB and Fosen KM: Effects of jasplakinolide on the kinetics of actin polymerization. An explanation for certain *in vivo* observations. *J Biol Chem* 275: 5163-5170, 2000.
- Moulding DA, Blundell MP, Spiller DG, White MRH, Cory GO, Calle Y, Kempinski H, Sinclair J, Ancliff PJ, Kinnon C, Jones GE and Thrasher AJ. Unregulated actin polymerization by WASp causes defects of mitosis and cytokinesis in X-linked neutropenia. *J Exp Med* 204: 2213-2224, 2007.
- Lieu C-H, Chang Y-N and Lai Y-K: Dual cytotoxic mechanisms of submicromolar taxol on human leukemia HL-60 cells. *Biochem Pharmacol* 53: 1587-1596, 1997.
- Buszek KR and Brown N: Improved method for the diimide reduction of multiple bonds on solid-supported substrates. *J Org Chem* 72: 3125-3128, 2007.
- Hamel E: Interaction of tubulin with small ligands. *In: Microtubule Proteins*. Avila J (ed.). Boca Raton, FL, CRC Press, pp. 89-191, 1990.

- 27 Schiff PB and Horwitz SB: Taxol assembles tubulin in the absence of exogenous guanosine 5'-triphosphate or microtubule-associated proteins. *Biochemistry* 20: 3247-3252, 1981.
- 28 Owellen RJ, Hartke CA, Dickerson RM and Hains FO: Inhibition of tubulin-microtubule polymerization by drugs of the vinca alkaloid class. *Cancer Res* 36: 1499-1502, 1976.
- 29 Goldbrunner M, Loidl G, Polossek T, Mannschreck A and von Angerer E: Inhibition of tubulin polymerization by 5,6-dihydroindolo[2,1- $\alpha$ ]isoquinoline derivatives. *J Med Chem* 40: 3524-3533, 1997.
- 30 Ben-Ze'ev A, Farmer SR and Penman S: Mechanisms of regulating tubulin synthesis in cultured mammalian cells. *Cell* 17: 319-325, 1979.
- 31 Shi Q and King RW: Chromosome nondisjunction yields tetraploid rather than aneuploid cells in human cell lines. *Nature* 437: 1038-1042, 2005.
- 32 Shimizu N, Itoh N, Utiyana H and Wahl GM: Selective entrapment of extrachromosomally amplified DNA by nuclear budding and micronucleation during S phase. *J Cell Biol* 140: 1307-1320, 1998.
- 33 Shimizu N, Shimuara T and Tanaka T: Selective elimination of acentric double minutes from cancer cells through the extrusion of MNi. *Mutat Res* 448: 81-90, 2000.
- 34 Pelham RJ and Chang F: Actin dynamics in the contractile ring during cytokinesis in fission yeast. *Nature* 419: 82-86, 2002.
- 35 Werner M and Glotzer M: Control of cortical contractility during cytokinesis. *Biochem Soc Trans* 36: 371-377, 2008.
- 36 Zabriskie TM, Klocke JA, Ireland CM, Marcus AH, Molinski TF, Faulkner DJ, Xu C and Clardy JC: Jaspamide, a modified peptide from a *Jaspis* sponge, with insecticidal and antifungal activity. *J Am Chem Soc* 108: 3123-3124, 1986.
- 37 Senderowicz AM, Kaur G, Sainz E, Laing C, Inman WD, Rodriguez J, Crews P, Malspeis L, Grever MR, Sausville EA and Duncan LK: Jaspalinolide's inhibition of the growth of prostate carcinoma cells *in vitro* with disruption of the actin cytoskeleton. *J Natl Cancer Inst* 87: 46-51, 1995.
- 38 Scott VR, Boehme R and Matthews TR: New class of antifungal agents: Jaspalinolide, a cyclodepsipeptide from the marine sponge, *Jaspis* species. *Antimicrob Agents Chemother* 32: 1154-1157, 1988.
- 39 Posey SC and Bierer BE: Actin stabilization by jaspalinolide enhances apoptosis induced by cytokine deprivation. *J Biol Chem* 274: 4259-4265, 1999.
- 40 Gohla A, Birkenfeld J and Bokoch GM: Chronophin, a novel HAD-type serine protein phosphatase, regulates cofilin-dependent actin dynamics. *Nature Cell Biol* 7: 21-29, 2005.
- 41 Westerberg LS, Meelu P, Baptista M, Eston MA, Adamovich DA, Cotta-de-Almeida V, Seed B, Rosen MK, Vandenberghe P, Thrasher AJ, Klein C, Alt FW and Snapper SB: Activating WASP mutations associated with X-linked neutropenia result in enhanced actin polymerization, altered cytoskeletal responses, and genomic instability in lymphocytes. *J Exp Med* 207: 1145-1152, 2010.
- 42 Moulding DA, Moeendarbary E, Valon L, Record J, Charras GT and Thrasher AJ: Excess F-actin mechanically impedes mitosis leading to cytokinesis failure in X-linked neutropenia by exceeding Aurora B kinase error correction capacity. *Blood* 120: 3803-3811, 2012.
- 43 DeWard AD and Alberts AS: Ubiquitin-mediated degradation of the formin mDia3 upon completion of cell division. *J Biol Chem* 284: 20061-20069, 2009.

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