Effects of the tumor inhibitory triterpenoid avicin G on cell integrity, cytokinesis, and protein ubiquitination in fission yeast

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Avicins comprise a class of triterpenoid compounds that exhibit tumor inhibitory activity. Here we show that avicin G is inhibitory to growth of the fission yeast *Schizosaccharomyces pombe*. *S. pombe* cells treated with a lethal concentration of avicin G (20 μM) exhibited a shrunken morphology, indicating that avicin G adversely affects cell integrity. Cells treated with a sublethal concentration of avicin G (6.5 μM) exhibited a strong cytokinesis-defective phenotype (multiseptated cells), as well as cell morphology defects. These phenotypes bear resemblance to those resulting from loss of Rho1 GTPase function in *S. pombe*. Indeed, Rho1-deficient *S. pombe* cells were strongly hypersensitive to avicin G, suggesting that the compound may perturb Rho1-dependent processes. Consistent with previously observed effects in human Jurkat T cells, avicin G treatment resulted in hyperaccumulation of ubiquitinated proteins in *S. pombe* cells. Interestingly, proteasome-defective *S. pombe* mutants were not markedly hypersensitive to avicin G, whereas an anaphase-promoting complex (mitotic ubiquitin ligase) mutant exhibited avicin G resistance, suggesting that the increase in levels of ubiquitinated proteins resulting from avicin G treatment may be due to increased protein ubiquitination, rather than inhibition of 26S proteasome activity. Mutants defective in the cAMP/PKA pathway also exhibited resistance to avicin G. Our results suggest that *S. pombe* will be a useful model organism for elucidating molecular targets of avicin G and serve as a guide to clinical application where dysfunctional aspects of Rho and/or ubiquitination function have been demonstrated as in cancer, fibrosis, and inflammation.

A major goal in clinical cancer research is to discover compounds that selectively inhibit the growth of cancer cells without adversely affecting normal cell growth. A series of studies with a recently discovered family of triterpenoid compounds, the avicins, have identified cancer and inflammatory diseases as potential clinical targets (1–6). Originally isolated from the Australian desert tree *Acacia victoriae*, avicins have been shown to selectively inhibit the growth of tumor cells from a wide variety of tissue origins (1–6). Moreover, avicin treatment resulted in a dramatic reduction in the frequencies of both *H-ras* mutations and aneuploidy in a mouse model for skin carcinogenesis (4), as well as decreased p53 mutations in a murine UVB skin model (3). The molecular mechanisms by which avicins inhibit tumor cell growth are not well defined. However, there is evidence that they affect multiple cellular processes, including activation of apoptosis and cellular stress response pathways, as well as growth factor signaling, inflammation, and oxidative stress response (1–6).

Elucidation of the underlying mechanisms by which avicins inhibit cell growth will greatly facilitate the rational designing and testing of improved drug analogs and could potentially result in the identification of novel targets for cancer intervention as well as new molecular markers for cancer diagnosis and prognosis. Because mammalian cells are not well suited for genetic screening approaches for pathway discovery, we sought to identify genetically tractable model eukaryotes that might serve this purpose. The fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae* were viewed as attractive candidates for our studies because of their amenability to genetic manipulation and because their genome sequences have been determined (7, 8). In preliminary experiments, we found that avicin G was significantly more cytotoxic to *S. pombe* than *S. cerevisiae*. Because of this finding, we decided to study further the effects of avicin G on *S. pombe* growth and physiology. In this paper, we show that avicin G is inhibitory to cell growth, integrity, and cytokinesis in *S. pombe*, and provide genetic evidence that it may act, in part, by perturbing Rho GTPase-dependent cellular processes. We also show that, similar to effects observed in human Jurkat T cells, avicin G strongly stimulates protein ubiquitination in *S. pombe* cells.

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Experimental Procedures

Yeast Strains and Manipulations. *S. pombe* strains used in this study were wild-type strains SP870 (*h*^{90} *ade6-210 leu1-32 ura4-D18*) (from D. Beach, University College London, London), SP870D (*h*^{90} *ade6-210 leu1-32 ura4-D18*/*h*^{90} *ade6-210 leu1-32 ura4-D18*) (9), and CHP428 (*h*$^{+}$ *ade6-M210 his7-366 leu1-32 ura4-D18*) (from C. Hoffman, Boston College, Boston); KS1366 (*spc1*Δ) (*h*$^{-}$ *leu1-32 ura4-D18 spc1*::*ura4*) (10), SPPLBU (*plb1*Δ) (*h*^{90} *ade6-210 leu1-32 ura4-D18 plb1*::*ura4*) (11), DY114 (*cyr1*Δ) (*h*^{90} *ade6-210 leu1-32 ura4-D18 cyr1*::*ura4*) (12), CHP453 (*pka1*Δ)(*h*$^{-}$ *leu1-32 ura4-D18 his7-366 pka1*::*ura4*) (from C. Hoffman), *mts2-1* (*h*$^{-}$ *leu1-32 ura4-D18 mts2-1*) (13), *mts3-1* (*h*$^{-}$ *leu1-32 mts3-1*) (13), *nuc2-663* (*h*$^{-}$ *leu1-32 nuc2-663*) (from E. Chang, Columbia University College of Physicians and Surgeons, New York), SPRHO1UD (*h*^{90} *ade6-210 leu1-32 ura4-D18*/*h*^{90} *ade6-210 leu1-32 ura4-D18 rho1*::*ura4*) (see below), and *nmt41-rho1* (*h*^{90} *ade6-210 leu1-32 ura4-D18 rho1*::*ura4 pREP41rho1*) (see below).

Standard yeast culture media and genetic methods were used (14, 15). *S. pombe* cultures were grown in either YEAU (0.5% yeast extract/3% dextrose/75 mg/liter adenine/75 mg/liter uracil) or synthetic minimal medium (Eagle's minimal medium, EMM) with appropriate supplements (14). Where indicated, avicin G was added to the growth medium.

Purification of Avicins. Avicins D and G (see Fig. 7, which is published as supporting information on the PNAS web site) were purified from *A. victoriae* root extracts as described (6).

Construction of the *nmt41-rho1* Strain. The PCR was used to amplify a 5′ end fragment of the *rho1* gene (*rho1F1*) using the oligonucleotide primers 5′-GGATAAGCGGATTAAATGCG and 5′-GCCAGTGGGATTTGTAGCTAAGCTTCCCT-AGATTTGTTTACTTTCTCCTGC, and a 3′ end fragment

Abbreviation: APC, anaphase-promoting complex.

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of *rho1* (*rho1F2*) was amplified by using the primers 5′-CAAAAAGTTTCGTCAATATCACAAGCTTCATCTC-TTCAGTCGAAGCACATT and 5′-GCTACTTCGACTAT-CAACGTG. The resulting *rho1F1* and *rho1F2* PCR products were used to amplify the *ura4* gene, thus generating a *rho1*::*ura4* PCR fragment in which the *rho1* protein coding sequence is deleted by the *ura4* gene. The *rho1*::*ura4* fragment was used to transform SP870D to create the $rho1^{+}/rho1$::*ura4* diploid strain, SPRHO1UD. SPRHO1UD was transformed with the plasmid pREP41rho1, which was constructed by cloning a PCR-derived fragment of the *rho1* protein coding sequence into the plasmid pREP41 (16). The resulting transformants were sporulated and subjected to random spore dissection to isolate a *rho1*::*ura4* haploid strains (*nmt41-rho1*) carrying the pREP41rho1 plasmid.

Avicin G Sensitivity Tests. Two types of assays were used to measure the sensitivity of *S. pombe* strains to avicin G. For the cell lawn sensitivity test (refer to Fig. 1*A*), *S. pombe* cultures were grown overnight in YEAU medium to $\approx 5 \times 10^6$ cells per ml. A total of 10^5 cells were then spread onto YEAU plates. Five-microliter volumes of avicin G (dissolved in water) were subsequently pipetted onto the cell lawns as indicated. For the cell spotting test (see Fig. 3*C*), *S. pombe* cultures were grown in YEAU or EMM, as indicated, to 5×10^6 cells per ml, washed once with growth medium, then resuspended at 5×10^6 cells per ml in growth medium. Dilution series (1:5) of the cultures were then prepared, and 3-μl volumes of the dilutions were spotted onto either YEAU or EMM plates containing avicin G as indicated.

Fluorescence Microscopy. *S. pombe* cells were stained with DAPI for visualization of nuclei by fluorescence microscopy as described (14).

Detection of Ubiquitinated Proteins. *S. pombe* cultures were lysed with glass beads in PEM buffer (100 mM Pipes/1 mM EGTA/1 mM $MgSO_4$, pH 6.9) containing 4 mM benzamidine, 10 μM E64, 50 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethanesulfonyl fluoride, and 2 μg/ml aprotinin essentially as described (17). Equal amounts of protein were resolved by SDS/PAGE and subsequent immunoblotting using anti-ubiquitin mouse monoclonal antibody (Stressgen Biotechnologies). For avicin G treatment experiments, cultures were grown overnight in YEAU to 5×10^6 cells per ml, at which point avicin G was added to the cultures.

Results

Avicin G Inhibits *S. pombe* Cell Growth at Micromolar Concentrations. As an initial step toward investigating the effects of avicin G on *S. pombe* cell growth, we overlaid 10^5 wild-type *S. pombe* cells on YEAU growth medium, then spotted 5-μl volumes of a 4-fold dilution series of avicin G (0.02–5 μg/μl) onto the cell lawn. The plates were then incubated for 3 days at 30°C to allow for growth of the cells. Significant growth inhibition (circular areas devoid of cell growth) was observed where 6.3- and 25-μg amounts of avicin G had been spotted, whereas a small area of growth inhibition resulted from a 1.6-μg application of the drug (Fig. 1*A*). These results show that avicin G has inhibitory effects on *S. pombe* cell growth. To determine the concentration of avicin G required for growth inhibition, liquid cultures of *S. pombe* cells were incubated in YEAU or YEAU containing concentrations of avicin G ranging from 5 μg/ml to 40 μg/ml. Cell growth was slightly inhibited by 5 μg/ml (2.5 μM) avicin G and strongly inhibited by 10 μg/ml of the drug (Fig. 1*B*). At 20 μg/ml and 40 μg/ml concentrations of avicin G, *S. pombe* cell growth was completely inhibited within 8 h of exposure to the drug (Fig. 1*B*). These results demonstrate that avicin G is inhibitory to *S. pombe* cell growth at micromolar concentrations.

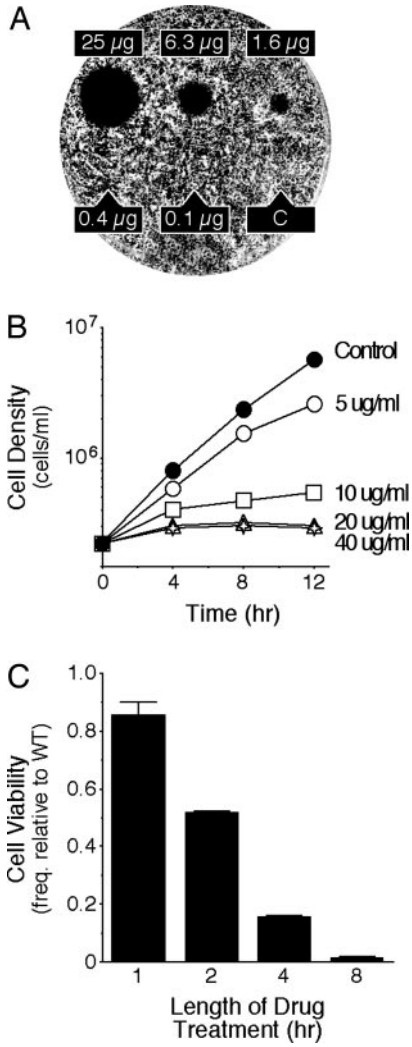

Fig. 1. Avicin G inhibits *S. pombe* cell growth. (*A*) Wild-type *S. pombe* cells (10^5) were spread on a YEAU plate. Five-microliter volumes of a 1:4 dilution series of avicin G (dissolved in water) were spotted onto the cell lawn (0.1–25 μg of drug), which were incubated for 3 days at 30°C. Five microliters of water was spotted on the plate as a control (C). Growth inhibition (circular areas devoid of cell growth) was observed where 1.6 μg, 6.3 μg, and 25 μg amounts of the drug were spotted. (*B*) Wild-type *S. pombe* cells were incubated in YEAU (control) or YEAU containing the indicated concentrations of avicin G and monitored for growth for 12 h. Cell densities (cells per ml) were determined microscopically by using a hemacytometer. (*C*) Wild-type *S. pombe* cells were incubated in YEAU to low density, then diluted with fresh YEAU to a density of 2.5×10^5 cells per ml. Avicin G was added to half the culture to a final concentration of 20 μg/ml, and an equal volume of water was added to the remaining culture. At 1, 2, 4, and 8 h, 10-ml portions of each culture were pelleted by centrifugation, washed twice with YEAU, and resuspended in YEAU. Cell densities were determined by using a hemacytometer, and 2,000 cells from each sample were spread onto YEAU plates. After 3 days, colonies were counted. The graph shows the frequency of cell viability (based on colony-forming units) of untreated and avicin G-treated cultures at the indicated time points. Duplicate samples were cultured for each time point.

To determine whether avicin G causes cell death or reversible inhibition of cell growth, *S. pombe* cells were incubated in YEAU or YEAU containing 20 μg/ml avicin G for 1, 2, 4, or 8 h. After drug treatment, the cells were washed with drug-free medium, diluted, and then plated onto YEAU plates to determine the frequency of viable cells as measured by colony formation. As shown in Fig. 1*C*, an $\approx$50% reduction in cell viability resulted after only 2 h of treatment with avicin G, and only a very small

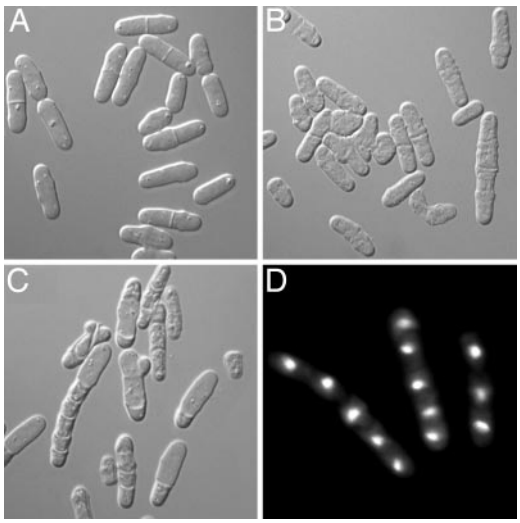

Fig. 2. Microscopic analysis of avicin G-treated *S. pombe* cells. Photomicrographs of wild-type *S. pombe* cells incubated in YEAU (*A*) or YEAU containing 40 μg/ml avicin G (*B*) for 10 h. The majority of avicin G-treated cells were shrunken in appearance. (*C*) Photomicrograph of wild-type *S. pombe* cells treated with a sublethal concentration of avicin G (10 μg/ml) for 10 h. A high frequency of cells contained multiple septa. (*D*) Fluorescence photomicrograph of DAPI-stained *S. pombe* cells treated with 10 μg/ml avicin G for 10 h. Three multiseptated cells are shown, the compartments of which each contained a single nucleus.

fraction of cells retained viability after 8 h of drug treatment. These results demonstrates that avicin G causes in cell death in *S. pombe*.

The second of two structurally defined avicin isoforms, avicin D (6), was also tested for effects on *S. pombe* cell growth. Avicin D was also found to be inhibitory to *S. pombe* cell growth, although its minimal inhibitory concentration was ≈5-fold higher than that of avicin G (data not shown). The difference in effectiveness between these two avicin isoforms toward *S. pombe* cells is similar to that noted previously in experiments analyzing the effects of avicins D and G on human Jurkat T cells (6). Avicin G was used for the remainder of this study because of the high cost associated with avicin purification.

Avicin G Causes Defects in *S. pombe* Cell Integrity and Cytokinesis. Microscopic analyses were performed to determine whether avicin G affects *S. pombe* morphology or cell division. *S. pombe* cells treated with a lethal concentration of avicin G (40 μg/ml) for 10 h were substantially shrunken in appearance in comparison to untreated control cells (Fig. 2 *A* and *B*). In cultures treated with a sublethal concentration of avicin G (10 μg/ml), we observed a high frequency of multiseptated cells (Fig. 2*C*), as well as a low frequency (<5%) of morphologically aberrant cells. Each compartment of multiseptated cells typically contained a single, well formed nucleus, even in cells with four or more septa (Fig. 2*D*). Taken together, these observations suggest that avicin G has adverse effects on cell integrity and that it is strongly inhibitory to a late step(s) in cytokinesis required for the completion of septation and/or cell separation.

Rho1 GTPase-Deficient *S. pombe* Cells Are Hypersensitive to Avicin G. The cell integrity-, cytokinesis-, and morphology-defective phenotypes caused by avicin G treatment bear resemblance to phenotypes caused by loss of function of the *S. pombe* Rho family GTPase, Rho1 (18, 19). If avicin G is inhibitory to Rho1-dependent processes in *S. pombe*, then we would expect cells deficient in Rho1 function to be hypersensitive to the drug. To

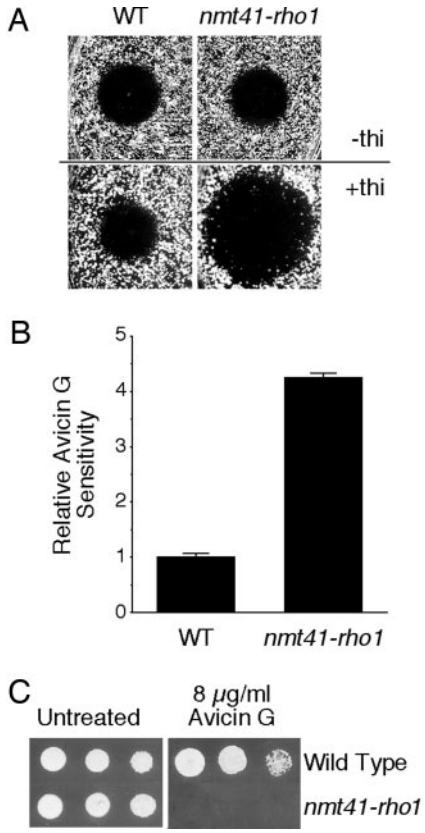

Fig. 3. Rho1-deficient *S. pombe* cells are hypersensitive to avicin G. (*A*) Wild-type and *nmt41-rho1* cells were overlaid onto EMM (*Upper*) or EMM plus 0.1 μM thiamine (EMMT) (*Lower*) as described for Fig. 1. Avicin G (25 μg) was spotted onto each cell lawn, and the plates were incubated for 3 days at 30°C. On EMMT plates, but not EMM plates, the area of avicin G-induced growth inhibition was significantly greater for *nmt41-rho1* lawns than the wild-type cell lawns. (*B*) Relative avicin G sensitivity of wild-type and *nmt41-rho1* cells based on areas of growth inhibition from two separate drug sensitivity tests of the type shown in *A*. Avicin G sensitivity of wild-type cells was normalized to a value of 1. (*C*) Serial dilutions (1:5) of wild-type and *nmt41-rho1* cells were spotted onto EMMT or EMMT containing 8 μg/ml avicin G and incubated for 5 days at 30°C. Wild-type cells grew on the avicin G plate, whereas *nmt41-rho1* cells did not.

determine whether this is the case, we constructed a *S. pombe* strain, *nmt41-rho1*, in which *rho1* expression is under the control of the thiamine repressible *nmt41* promoter (16). The *nmt41-rho1* strain is inhibited for growth in media containing 50 μM thiamine, whereas in media containing 0.1 μM thiamine, it is phenotypically similar to wild-type cells (data not shown). Wild-type and *nmt41-rho1* cultures were grown in EMM medium to mid-log phase, then 10^5 cells from each culture were overlaid onto EMM or EMM containing 0.1 μM thiamine (EMMT). Avicin G (25 μg) was then spotted onto each cell lawn and the plates were incubated for 3 days at 30°C to allow for growth of the cells. On EMM plates lacking thiamine, wild-type and *nmt41-rho1* strains exhibited similar sensitivities to avicin G (Fig. 3*A Upper*). However, on EMMT plates, the area of avicin G-induced growth inhibition was significantly greater for *nmt41-rho1* cells than wild-type cells (Fig. 3 *A Lower* and *B*). These results provide evidence that Rho1-deficient *S. pombe* cells are hypersensitive to avicin G. This conclusion was supported by the results of a second type of drug sensitivity test in which serial dilution series of wild-type and *nmt41-rho1* cultures were spotted onto EMMT or EMMT containing a concentration of avicin G (8 μg/ml) that is partially inhibitory to wild-type cell growth.

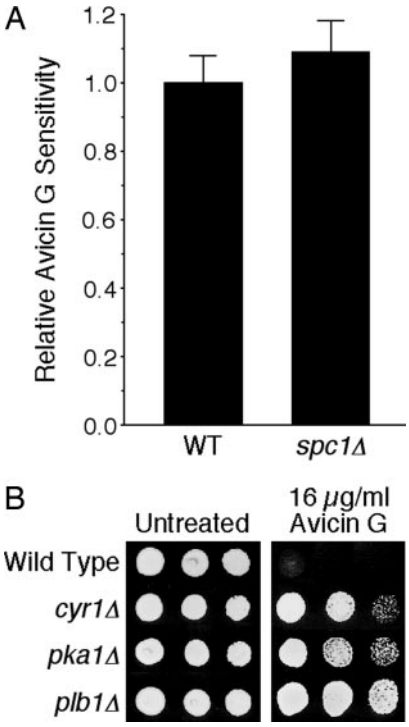

Fig. 4. Relative avicin G sensitivities of stress-response-defective *S. pombe* mutants. (*A*) Wild-type and *spc1*Δ *S. pombe* strains were overlaid onto YEAU plates as described in Fig. 1. Avicin G (25 μg) was then spotted onto the respective cell lawns, and the plates were incubated at 30°C for 3 days. The relative avicin G sensitivity of each strain, based on measurements of areas of avicin G-induced growth inhibition, was then determined, with wild-type cells being normalized to a value of 1. (*B*) Serial dilutions (1:5) of wild-type, *cyr1*Δ, *pka1*Δ, and *plb1*Δ cells were spotted onto YEAU or YEAU containing 16 μg/ml avicin G and incubated for 3 days at 30°C. *cyr1*Δ, *pka1*Δ, and *plb1*Δ cells, but not wild type, grew on the avicin-G-containing plate.

Wild-type and *nmt41-rho1* cells grew equally well on EMMT (Fig. 3*C Left*). However, whereas wild-type cells grew on EMMT containing 8 μg/ml avicin G, *nmt41-rho1* cells were completely inhibited for growth on the same medium (Fig. 3*C Right*).

Effects of Avicin G on the Growth of Stress-Response-Defective *S. pombe* Mutants. We recently showed that in mammalian cells avicin G induces expression of a gene battery that enhances protection against oxidative and other stresses (3). In *S. pombe*, the mitogen-activated protein kinase homolog, Spc1, is an essential mediator of several forms of environmental stress, including not only oxidative, but also osmotic, heat, UV, and nutrient stresses (20). We found that *spc1*Δ cells were only slightly more sensitive to avicin G than wild-type *S. pombe* cells (Fig. 4*A*). This finding suggests that avicin G treatment does not likely induce physiological stresses of the type that are normally mediated by the Spc1 pathway. We also tested the effects of avicin G on strains carrying null mutations in genes encoding adenylate cyclase (*cyr1*), protein kinase A (*pka1*), and phospholipase B1 (*plb1*), each of which is required for growth of *S. pombe* cells in high-osmolarity media (11). Interestingly, each of these mutants exhibited markedly reduced sensitivity to avicin G in comparison to wild-type *S. pombe* cells (Fig. 4*B*). This finding suggests that loss of function of the Cyr1, Pka1, and Plb1 proteins may result in physiological defects that are partially protective against the cytotoxic effects of avicin G.

Avicin G Causes Up-Regulation of Protein Ubiquitination in *S. pombe* Cells. We recently showed that avicin G treatment results in hyperaccumulation of ubiquitinated proteins in human Jurkat T cells (21). Because of this finding, experiments were carried out to determine whether avicin G also affects protein ubiquitination in *S. pombe* cells. Wild-type *S. pombe* cells were treated with 20 μg/ml avicin G, and aliquots of the cell cultures were harvested between 30 min and 4 h after exposure to the drug. Cell extracts were then prepared and resolved by SDS/PAGE and subsequent immunoblotting to detect ubiquitinated proteins. As shown in Fig. 5*A*, an increase in ubiquitinated proteins was apparent after 90 min of avicin G treatment, and the levels of ubiquitinated proteins increased significantly with prolonged drug treatment. This result demonstrates that, similar to its effects in Jurkat T cells, avicin G treatment induces a dramatic increase in the levels of ubiquitinated proteins in *S. pombe* cells.

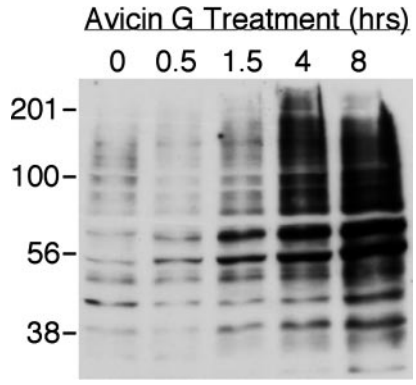

Fig. 5. Avicin G causes hyperaccumulation of ubiquitinated proteins in *S. pombe* cells. Wild-type *S. pombe* cells were incubated in YEAU containing 20 μg/ml avicin G for the time indicated (h), then processed for immunoblot analysis of ubiquitinated proteins. An increase in the levels of ubiquitinated proteins was detected after 1.5 h of avicin G treatment.

Because *S. pombe* cells deficient in Rho1 function are hypersensitive to avicin G, we carried out experiments to examine whether loss of Rho1 function results in increased protein ubiquitination in *S. pombe*. Wild-type and *nmt41-rho1* cells were incubated in 50 μM thiamine for 9 h, which results in repression of *rho1* expression from the *nmt1* promoter in the latter strain. Cell lysates were prepared and analyzed by SDS/PAGE and immunoblotting to detect ubiquitinated proteins. Wild-type and *nmt41-rho1* cells had similar levels of ubiquitinated proteins (data not shown), indicating that loss of Rho1 function, unlike avicin G treatment, does not result in increased protein ubiquitination in *S. pombe*.

A *S. pombe* Mutant Defective in Function for the Anaphase-Promoting Complex (APC) Exhibits Marked Resistance to Avicin G. We used *S. pombe* mutant strains to investigate whether the increase in levels of ubiquitinated proteins resulting from avicin G treatment might be attributable to inhibition of 26S proteasome activity, up-regulation of protein ubiquitination, or both. Two temperature-sensitive 26S proteasome mutants, *mts2-1* and *mts3-1* (13), were found to exhibit sensitivities to avicin G that were only slightly increased from wild-type *S. pombe* cells at their semipermissive growth temperature of 26°C (Fig. 6*A*). In contrast to this result, we found that a *S. pombe* mutant carrying a temperature-sensitive mutation in the *nuc2* gene (*nuc2-663*), which encodes an essential component of the APC mitotic ubiquitin ligase complex in *S. pombe* (22), was markedly resistant to avicin G (Fig. 6). These results suggest that the increase in levels of ubiquitinated proteins that occurs in response to avicin G treatment may be attributable to up-regulation of protein ubiquitination, rather than to inhibition of 26S proteasome activity, an experimental conclusion similar to that achieved with human leukemia cells (21).

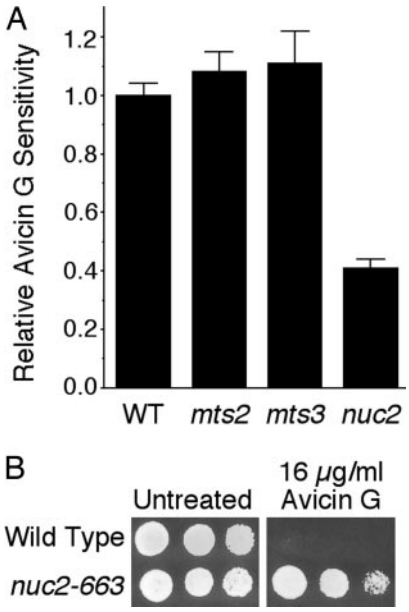

Fig. 6. Effects of avicin G on the growth of 26S proteasome and APC-defective *S. pombe* mutants. Wild-type, *mts2-1* (*mts2*), *mts3-1* (*mts3*), and *nuc2-663* cells were spread onto a YEAU plates as described for Fig. 1. Avicin G (25 μg) was then spotted onto the respective cell lawns, and the plates were incubated at 26°C for 5 days. The relative avicin G sensitivity of each strain, based on measurements of areas of avicin G-induced growth inhibition, was then determined, with wild-type cells being normalized to a value of 1. (*B*) Serial dilutions (1:5) of wild-type and *nuc2-663* cells were spotted onto YEAU or YEAU containing 16 μg/ml avicin G and incubated for 5 days at 26°C. *nuc2-663* cells, but not wild-type cells, grew on the avicin G-containing plate.

Discussion

Triterpenoids are among the most common metabolites produced by many different plant species, as well as certain bacteria and lower marine animals (23). Triterpenoids, which contain 30 carbons consisting of five carbon isoprene units, have been shown to possess antimicrobial, antiinflammatory, and antineoplastic activities (24). Therefore, they are viewed with great interest with respect to their potential medicinal values. The precise underlying mechanisms by which triterpenoids affect cell physiology are, at present, largely unknown. The avicins are a structurally unique class of triterpenoid compounds in that they contain an electrophilic C-21 side chain (6).

The avicins have been shown to affect multiple cellular processes. For example, perturbation of the mitochondria by avicins initiates the apoptotic response (2). Activation of the ubiquitin pathway by avicins is responsible for removing post-mitochondrial barriers to apoptosis (unpublished data). Inhibition of inflammation and activation of stress and oxidant defense are controlled by redox regulation of transcription factors (1, 3). The latter activity appears to be secondary to a reversible transesterification of critical cysteines by avicins (25). Inhibition of growth factor signaling and stress regulation are also controlled by avicin's ability to regulate signal transduction, possibly due to activation of a G protein-coupled receptor (5, 21).

Future development of avicins and their potential derivatives as therapeutic agents for disease intervention will benefit from the elucidation of the underlying mechanisms responsible for their cytotoxic, as well as cytoprotective, effects. Therefore, we investigated whether the genetically tractable fission yeast might be useful as a model organism for gaining insights into the mechanisms by which avicins inhibit cell growth. Avicin G was found to be cytotoxic to *S. pombe* at micromolar concentrations. A structurally similar isoform of the drug, avicin D, was also inhibitory to *S. pombe* growth, but was ≈5-fold less effective than avicin G. *S. pombe* cells treated with a lethal concentration of avicin G (20 μM) were shrunken in appearance, suggesting that the drug adversely affects cell integrity. In cultures treated with a sublethal concentration of avicin G (5 μM), we observed high frequencies of multiseptated cells, as well as lower frequencies of morphologically aberrant cells. We did not detect obvious chromosome segregation defects in avicin G treated cultures, nor was there evidence of nuclear cutting by the septum ("cut" phenotype) (26) in dividing cells. Taken together, our findings suggest that avicin G is inhibitory to cell integrity and to a late step in cytokinesis required for efficient cell separation.

The observed effects of avicin G bear resemblance to phenotypes caused by loss of Rho1 function in *S. pombe* (18, 19). Indeed, we found that Rho1-deficient *S. pombe* cells were strongly hypersensitive to avicin G, suggesting that the drug may perturb Rho1-dependent processes. Future studies will investigate whether avicin G affects Rho1 activity, either by affecting GTP binding or isoprenylation of the Rho1 protein. Certain classes of saponins have been shown to form complexes with cholesterol (27, 28), and in laboratory animals, this can lead to changes in cholesterol metabolism (29). Therefore, one speculation is that avicin G might affect the ergosterol (the fungal equivalent of cholesterol) biosynthesis pathway in *S. pombe*, and, as a result, alter the metabolism of isoprenoid intermediates generated by the pathway, which are used in protein prenylation. An alternative mechanism could be the inhibition of prenylation of Rho by avicins. This latter possibility is strengthened by posttranslational effects of avicins on thiol-containing proteins (1, 3), as well as the known effect of monoterpenes, which are present in the avicin side chain, on prenylation (30).

We recently showed that, in human Jurkat T cells, avicin G treatment results in increased levels of ubiquitinated proteins (21). This effect of the compound appears to be due, at least in part, to up-regulation of ubiquitin ligase activity. We have shown here that avicin G treatment also results in hyperaccumulation of ubiquitinated proteins in *S. pombe* cells and that a mutant defective in function for the APC mitotic ubiquitin ligase complex is markedly resistant to avicin G. Taken together, these findings suggest that an apparently conserved effect of avicin G is to induce up-regulation of protein ubiquitination. Rho1-deficient *S. pombe* cells were found to have similar levels of ubiquitinated proteins as wild-type cells, demonstrating that loss of Rho1 function, unlike avicin G treatment, does not lead to changes in protein ubiquitination. Therefore, it seems likely that avicin G has more than one molecular target in *S. pombe* cells.

We have previously shown that avicin enhances the nuclear localization and transcriptional activation of Nrf2, a redox-regulated transcription factor that regulates the expression of a number of genes encoding proteins required for cellular response to oxidative stress (3, 31, 32). In the present study, we determined that *S. pombe* cells carrying a deletion of the *spc1* gene, which encodes a mitogen-activated protein kinase required for survival of oxidative and other extracellular stresses in *S. pombe* (12), exhibited a similar sensitivity to avicin G as that observed for wild-type cells. This result suggests that avicin G does not induce general physiological stresses of the type that are mediated by the Spc1 pathway. Interestingly, *S. pombe* adenylate cyclase (*cyr1*Δ), protein kinase A (*pka1*Δ), and phospholipase B (*plb1*Δ) mutants all exhibited marked resistance to avicin G. The Cyr1, Pka1, and Plb1 proteins are required for growth of *S. pombe* cells under hyperosmotic conditions, and the results of genetic experiments suggest that they may function in a common pathway (11). The results presented here suggest that loss of function of this pathway results in changes in cell physiology that are cytoprotective against avicin G. It is unclear what aspects of cell physiology might be altered. However, the involvement of a phospholipase (Plb1) raises the possibility that the avicin G may

affect phospholipid-dependent processes. Thus, the cytotoxic as well as cytoprotective effects of avicins in *S. pombe* cells with various mutations promise to further dissect the pathways responsible for the bifunctional effects of avicins: proapoptotic, as well as cytoprotective (3, 33).

In conclusion, the findings of this study demonstrate that *S. pombe* will serve an important role as a genetically tractable model organism for elucidation of the underlying, potentially conserved mechanisms of avicin G-induced cytotoxicity. Our study has identified potential pathways that might be affected by avicin G, and the effects of the compound on specific proteins in these pathways (e.g., Rho1 and APC) will need to be investigated in future studies. We have also isolated avicin G resistant *S. pombe* mutants in preliminary studies, and the characterization of these mutants will undoubtedly provide important insights into the diversity of physiological pathways affected by avicin G as well as potential molecular targets of the drug. Finally, these results have potentially important implications for clinical application. For example, activation as well as loss of Rho function are frequently observed in various cancer cells, as well as in profibrotic conditions (34–39). Activation of the ubiquitin–proteasome pathway has important implications for cancer and vascular and inflammatory conditions where dysfunctional ubiquitination has been described (40).

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1. Haridas, V., Arntzen, C. J. & Gutterman, J. U. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 11557–11562.
2. Haridas, V., Higuchi, M., Jayatilake, G. S., Bailey, D., Mujoo, K., Blake, M. E., Arntzen, C. J. & Gutterman, J. U. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 5821–5826.
3. Haridas, V., Hanausek, M., Nishimura, G., Soehnge, H., Gaikwad, A., Narog, M., Spears, E., Zoltaszek, R., Walaszek, Z. & Gutterman, J. U. (2004) *J. Clin. Invest.* **113,** 65–73.
4. Hanausek, M., Ganesh, P., Walaszek, Z., Arntzen, C. J., Slaga, T. J. & Gutterman, J. U. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 11551–11556.
5. Mujoo, K., Haridas, V., Hoffmann, J. J., Wachter, G. A., Hutter, L. K., Lu, Y., Blake, M. E., Jayatilake, G. S., Bailey, D., Mills, G. B. & Gutterman, J. U. (2001) *Cancer Res.* **61,** 5486–5490.
6. Jayatilake, G. S., Freeberg, D. R., Liu, Z., Richheimer, S. L., Blake Nieto, M. E., Bailey, D. T., Haridas, V. & Gutterman, J. U. (2003) *J. Nat. Prod.* **66,** 779–783.
7. Johnston, M. (1996) *Curr. Biol.* **6,** 500–503.
8. Wood, V., Gwilliam, R., Rajandream, M. A., Lyne, M., Lyne, R., Stewart, A., Sgouros, J., Peat, N., Hayles, J., Baker, S., *et al.* (2002) *Nature* **415,** 871–880.
9. Marcus, S., Polverino, A., Chang, E., Robbins, D., Cobb, M. H. & Wigler, M. H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6180–6184.
10. Shiozaki, K. & Russell, P. (1995) *Nature* **378,** 739–743.
11. Yang, P., Du, H., Hoffman, C. S. & Marcus, S. (2003) *Mol. Genet. Genomics* **269,** 116–125.
12. Kawamukai, M., Ferguson, K., Wigler, M. & Young, D. (1991) *Cell Regul.* **2,** 155–164.
13. Seeger, M., Gordon, C., Ferrell, K. & Dubiel, W. (1996) *J. Mol. Biol.* **263,** 423–431.
14. Alfa, C., Fantes, P., Hyams, J., McLeod, M. & Warbrick, E. (1993) *Experiments with Fission Yeast*: *A Laboratory Course Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
15. Rose, M. D., Winston, F. & Hieter, P. (1990) *Methods in Yeast Genetics*: *A Laboratory Course Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
16. Maundrell, K. (1990) *J. Biol. Chem.* **265,** 10857–10864.
17. Yen, H. C., Espiritu, C. & Chang, E. C. (2003) *J. Biol. Chem.* **278,** 30669–30676.
18. Nakano, K., Arai, R. & Mabuchi, I. (1997) *Genes Cells* **2,** 679–694.
19. Arellano, M., Duran, A. & Perez, P. (1997) *J. Cell Sci.* **110,** 2547–2555.
20. Degols, G., Shiozaki, K. & Russell, P. (1996) *Mol. Cell. Biol.* **16,** 2870–2877.
21. Gaikwad, A., Poblenz, A., Haridas, V., Zhang, C., Duvic, M. & Gutterman, J. (2005) *Clin. Cancer Res.* **11,** 1953–1962.
22. Yamada, H., Kumada, K. & Yanagida, M. (1997) *J. Cell Sci.* **110,** 1793–1804.
23. Hostettmann, M. & Marston, A. (1995) *Chemistry and Pharmacology of Natural Products*: *Saponins* (Cambridge Univ. Press, Cambridge, U.K.).
24. Francis, G., Kerem, Z., Makkar, H. P. & Becker, K. (2002) *Br. J. Nutr.* **88,** 587–605.
25. Valsala Haridas, V., Kim, S.-O., Nishimura, G., Hausladen, A., Stamler, J. S. & Gutterman, J. U. (2005) *Proc. Natl. Acad. Sci. USA* **102,** 10088–10093.
26. Yanagida, M. (1998) *Trends Cell Biol.* **8,** 144–149.
27. Wulff, G. (1968) *Dtsch. Apoth. Ztg.* **108**, 797–808.
28. Tschesche, R. & Wulff, G. (1964) *Planta Medica* **12,** 272–292.
29. Oakenfull, D. G. & Topping, D. L. (1983) *Atherosclerosis* **48,** 301–303.
30. Harrewijn, P., Van Oosten, A. M. & Piron, P. G. M. (2001) *Natural Terpenoids as Messengers*: *A Multidisciplinary Study of Their Production, Biological Functions and Practical Applications* (Kluwer Academic, Amsterdam, The Netherlands).
31. Itoh, K., Ishii, T., Wakabayashi, N. & Yamamoto, M. (1999) *Free Radic. Res.* **31,** 319–324.
32. Venugopal, R. & Jaiswal, A. K. (1998) *Oncogene* **17,** 3145–3156.
33. Blackstone, N. W., Kelly, M. M., Haridas, V. & Gutterman, J. U. (2005) *Proc. Biol. Sci.* **272,** 527–531.
34. Malliri, A. & Collard, J. G. (2003) *Curr. Opin. Cell Biol.* **15,** 583–589.
35. Rees, R. W., Foxwell, N. A., Ralph, D. J., Kell, P. D., Moncada, S. & Cellek, S. (2003) *J. Urol.* **170,** 2517–2522.
36. Boettner, B. & Van Aelst, L. (2002) *Gene* **286,** 155–174.
37. Adam, R. (2003) *J. Urol.* **170,** 2523–2524.
38. Shimizu, Y., Dobashi, K., Iizuka, K., Horie, T., Suzuki, K., Tukagoshi, H., Nakazawa, T., Nakazato, Y. & Mori, M. (2001) *Am. J. Respir. Crit. Care Med.* **163,** 210–217.
39. Mallat, Z., Gojova, A., Sauzeau, V., Brun, V., Silvestre, J. S., Esposito, B., Merval, R., Groux, H., Loirand, G. & Tedgui, A. (2003) *Circ. Res.* **93,** 884–888.
40. Glickman, M. H. & Ciechanover, A. (2002) *Physiol. Rev.* **82,** 373–428.