Published in final edited form as:
Yeast. 2005 September ; 22(12): 971–978. doi:10.1002/yea.1284.

Paclitaxel-induced microtubule stabilization causes mitotic block and apoptotic-like cell death in a paclitaxel-sensitive strain of *Saccharomyces cerevisiae*

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Abstract

Wild-type *Saccharomyces cerevisiae* tubulin does not bind the anti-mitotic microtubule stabilizing agent paclitaxel. Previously, we introduced mutations into the *S. cerevisiae* gene for β-tubulin that imparted paclitaxel binding to the protein, but the mutant strain was not sensitive to paclitaxel and other microtubule-stabilizing agents, due to the multiple ABC transporters in the membranes of budding yeast. Here, we introduced the mutated β-tubulin gene into a *S. cerevisiae* strain with diminished transporter activity and developed the first paclitaxel-sensitive budding yeast strain. In the presence of paclitaxel, cytoplasmic microtubules were stable to cold depolymerization. Paclitaxel-treated cells showed evidence of a mitotic block, with an increase in large-budded cells and cells with a 2N DNA content and DNA fragmentation, identified by FACS analysis and the TUNEL assay. In the presence of paclitaxel, the number of dead cells in cultures increased three-fold and cells containing reactive oxygen species were present. We conclude that paclitaxel blocks mitosis in this strain, leading to an apoptotic-like cell death. This strain will also be useful in further studies of the effect of microtubule dynamics on various cellular processes in *S. cerevisiae*.

Keywords

paclitaxel; tubulin; microtubules; yeast; apoptosis

Introduction

Paclitaxel (Taxol™) is an anti-tumour agent that has proved to be effective against a number of cancers. Its primary mechanism of action in cells is to cause a mitotic block by stabilizing microtubules, thereby decreasing the dynamic nature of these cytoskeletal structures (Jordan, 2002). Prolonged exposure of mammalian cells to paclitaxel leads to apoptotic cell death (Woods *et al.*, 1995). Microtubules are composed of the protein tubulin, a heterodimer consisting of α- and β-subunits. Paclitaxel binds to the β-subunit of tubulin in a 1 : 1 stoichiometry (Parness and Horwitz, 1981). It is essential that the binding interactions between the drug and tubulin be defined in detail so that rational drug design can be used to create more efficient paclitaxel derivatives. Although a high-resolution X-ray crystallographic structure of the tubulin–paclitaxel complex is lacking, information from a 3.7 Å resolution electron microscopic crystal structure (Nogales *et al.*, 1998), photoaffinity

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labelling experiments (Combeau *et al.*, 1994; Dasgupta *et al.*, 1994; Rao *et al.*, 1994, 1995, 1999), studies using NMR, fluorescence and molecular modelling (He *et al.*, 2000; Li *et al.*, 2000; Snyder *et al.*, 2001) and paclitaxel-resistant cell lines (Giannakakou *et al.*, 1997, 2000; Gonzalez-Garay *et al.*, 1999) have been used to identify the significance of specific amino acid residues in paclitaxel binding.

Growth of *Saccharomyces cerevisiae* (budding yeast) is not inhibited by paclitaxel and purified yeast tubulin does not interact with paclitaxel (Kilmartin, 1981; Barnes *et al.*, 1992; Bode *et al.*, 2002). In an investigation into the lack of paclitaxel binding to yeast tubulin, we identified five residues implicated in paclitaxel binding to mammalian tubulin that differ between yeast and mammalian tubulin (Gupta *et al.*, 2003). We provided experimental evidence for the importance of these five residues by mutating the residues in *S. cerevisiae* tubulin to those that occur in brain tubulin and showing that the mutated tubulin was capable of binding paclitaxel *in vitro* (Gupta *et al.*, 2003). Because creating mutations in yeast is straightforward, we can now use our mutated yeast tubulin to probe more deeply into the interactions between tubulin and paclitaxel, e.g. we can determine the relative importance of the five amino acids already mutated and the role of other amino acids in the binding interactions.

Structure–activity relationship studies incorporating large numbers of paclitaxel analogues in conjunction with modified paclitaxel binding sites would be greatly aided by a simple, cell-based assay as a primary screen to assess tubulin-paclitaxel interactions. Unfortunately, proliferation of budding yeast containing the mutated tubulin is not inhibited by paclitaxel, most likely because yeast contain multiple ABC transporters that prevent a number of xenobiotics from accumulating in the cell (Decottignies and Goffeau, 1997). As described in this article, we have solved this problem by placing the mutated β-tubulin gene into a strain with diminished ABC-mediated transport (Decottignies *et al.*, 1998), producing a strain that arrests in mitosis and undergoes apoptotic-like cell death in the presence of paclitaxel. This strain will be a valuable tool for the development of cell-based assays to assess tubulin–paclitaxel interactions and studies of microtubule dynamics on a variety of yeast cellular functions.

Materials and methods

Yeast strains and media

Strain MGY1-tax (genotype: *MAT***a**, *leu2Δ1, trpΔ63, his4-917, URA3/ura3-52, tub2-His*$_6$*-A19K-T23V-G26D-N227H-Y270F*) contains five mutations in the β-tubulin gene that impart paclitaxel binding activity by tubulin and was derived from strain MGY1 (genotype: *MAT*a, *leu2Δ1, trpΔ63, his4-917, URA3/ura3-52, tub2-His*$_6$; Gupta *et al.*, 2003). Strain AD 12 345 678 (AD1-8), in which the genes for seven ABC transporters and a transporter transcription factor have been inactivated, was obtained from André Goffeau, Université Cathlique de Louvain, Louvain-la-Neuve, Belgium, and has the genotype *MATα, PDR1-3, ura3, his1, Δyor1::hisG, Δsnq2::hisG, Δpdr5::hisG, Δpdr10::hisG, Δpdr11::hisG, Δycf1::hisG, Δpdr3::hisG, Δpdr15::hisG* (Decottignies *et al*, 1998). Strain AD1-8 was transformed with a DNA fragment containing the *tub2-His*$_6$*-A19K-T23V-G26D-N227H-Y227F* gene to produce strain AD1-8-tax using procedures described previously (Gupta *et al.*, 2001). The yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30 °C. The transformant AD1-8-tax was selected in SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose and supplemented with histidine).

Proliferation assays

The cell proliferation assays were performed on agar plates and in liquid medium. Yeast strains MGY1, MGY1-tax, AD1-8 and AD1-8-tax were incubated overnight in YPD medium at 30 °C to stationary phase. A sample of each culture was serially diluted in 10-fold steps up to a 10^6 dilution. Each dilution was spotted on YPD-agar plates containing 0, 15 and 25 μM paclitaxel using a Frogger (Dan-Kar Corp., Reading, MA) and the plates were incubated at 30 °C for 3 days.

The proliferation assays in liquid medium to determine IC_{50} values were performed in a final volume of 120 μl in sterile 96-well plates. Each well, containing 100 μl YPD supplemented with 100 U penicillin, 100 μg streptomycin, and paclitaxel in the range 0–25 μM, was inoculated with 2000 cells in a 20 μl volume from an overnight culture. The plates were placed in a humidified chamber on a shaker and incubated at 30 °C for 48 h. The plates were then agitated on a vortex plate shaker to ensure that yeast cells were completely suspended in solution and the optical density was read at 610 nm in a 96-well plate reader (Bio-tek Instruments, Inc.). Experiments were done in triplicate.

FACS analysis

Asynchronous log-phase cultures of MGY-1-tax, AD1-8 and AD1-8-tax were incubated either in the absence of paclitaxel or in the presence of 25 μM paclitaxel. Each culture was fixed and stained with propidium iodide using a procedure similar to one described by Hutter and Eipel (1978). Flow cytometry was performed on a Becton Dickinson FACScan and analysed using Cell Quest software. For each experiment at least 10 000 cells were analysed.

Bud morphology

To determine bud morphology distributions, log-phase cells were grown for 6 h with and without 25 μM paclitaxel. The cultures were sonicated briefly and 10 μl of each culture was mounted on a microscope slide. At least 200 cells in each culture were classified as non-budded, small-budded or large-budded (>50% of mother cell diameter).

Microscopy

To visualize microtubules, log-phase cultures were incubated at 30 °C in the presence of 25 μM paclitaxel for 6 h. A portion of the cells from the cultures was fixed using the method of Pringle and Hartwell (1981). The remaining cells were incubated at 4 °C for 20 h before fixation. The primary antibody used was the anti-α-tubulin antibody YOL1/34 and the secondary antibody was a fluorescein-conjugated goat anti-rat antibody; both were purchased from Accurate Chemical and Scientific Corp., Westbury, NY, and were used at a 1 : 250 dilution. The cells were visualized using a 63×/1.4NA planapochromat objective lens on a Ziess Axioplan 2ie microscope equipped for epifluorescence and transmitted light DIC microscopy. A Hamamatsu Orca-ER CCD camera linked to Open-lab software was used to capture images.

Programmed cell death assays

Log-phase cultures of AD1-8 and AD1-8-tax were analysed for reactive oxygen species (ROS) production, using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes, Eugene, OR) and for dead cells using phloxine B staining, as described by Severin and Hyman (2002). A modified form of the TUNEL assay, described by Roche Applied Sciences, Indianapolis, IN, was used to determine DNA degradation. In this modified assay the secondary anti-fluorescein antibody was not used and fluorescence microscopy was used to detect the fluorescein-labelled DNA directly

(Pozniakovsky *et al.*, 2005). For the TUNEL assay the cells were treated as described by Kilmartin and Adams (1984) before the addition of the TUNEL reagents.

Results

Inhibition of cell proliferation by paclitaxel

Because MGY1-tax, the strain containing the mutated β-tubulin gene, is not sensitive to paclitaxel, we introduced the mutated gene into strain AD1-8 (Decottignies *et al.*, 1998), in which the genes for seven ABC transporters and a transporter transcription factor have been inactivated. We tested the paclitaxel sensitivity of the new strain, AD1-8-tax, using two different assays. In one assay, cells at increasing dilutions were spotted onto agar plates with and without the drug (Figure 1). The proliferation of MGY1, MGY1-tax and AD1-8 is not affected by up to 25 μM paclitaxel, but paclitaxel clearly inhibited proliferation of the AD1-8-tax strain. When the cells were assayed in liquid medium, the IC_{50} value was found to be 6 μM (Figure 2).

Paclitaxel-induced microtubule stability

Cell proliferation of strain AD1-8, with diminished ABC transporter activity, was not inhibited by paclitaxel but, after mutating the gene for β-tubulin in this strain to create paclitaxel binding, the drug inhibited cell proliferation. Therefore, proliferation inhibition is most likely a result of paclitaxel-induced microtubule stabilization. To test this hypothesis, cells were incubated with 25 μM paclitaxel for 20 h at 4 °C and examined for microtubules using immunofluorescence microscopy (Figure 3). The 4 °C treatment caused the disappearance of microtubules in the AD1-8 strain, but in the AD1-8-tax strain, microtubules were still present. The mean length of the cytoplasmic microtubules after 20 h at 4 °C was twice that for microtubules in cells not incubated in the cold (n = 220).

Paclitaxel-induced mitotic block

As an anti-mitotic agent, paclitaxel would be expected to inhibit cell proliferation at the G_2/M phase of the cell cycle, leading to the build-up of cells having a 2N DNA content (the AD1-8-tax strain is haploid) and an accumulation of large-budded cells. *S. cerevisiae* cells are classified by bud morphology as unbudded (G_1 phase), small-budded (S phase) and large-budded (G_2/M phase). As shown in Figure 4, after 6 h of growth in the presence of 25 μM paclitaxel, the proportion of large-budded cells increased from 24% (without paclitaxel) to 58% (with paclitaxel) of the population. Unbudded cells decreased from 44% to 19% in the presence of paclitaxel. FACS analysis of the DNA content after treatment with paclitaxel for 6 h showed that, in the AD1-8-tax culture, the percentage of 2N DNA cells increased at the expense of 1N cells (Figure 5). Paclitaxel had no effect on the distribution of 1N and 2N cells in the AD1-8 and MGY1-tax cultures. Paclitaxel also caused the appearance of a sub-1N peak in the FACS profile of the AD1-8-tax cultures, indicative of DNA fragmentation in some cells. After 24 h in the presence of paclitaxel, the cultures were approaching stationary phase and 1N cells were the dominant species in the MGY1-tax and AD1-8 cultures. In the AD 1-8-tax culture 2N, and sub-1N DNA content predominated. The presence of fragmented DNA suggests that cells are undergoing an apoptotic-like death.

Paclitaxel-induced apoptosis

The appearance of cells with a DNA content less than 1N in the FACS analysis suggests that paclitaxel induced apoptosis. We therefore examined the cultures for indicators of programmed cell death using phloxine B staining for cell death, the TUNEL assay for DNA fragmentation, and staining for the presence of reactive oxygen species (ROS). Phloxine B is accumulated by dead but not by live cells. The results in Figure 6 show that, after 6 h in the

presence of 25 μM paclitaxel, the percentage of AD1-8-tax cells stained by phloxine B increased by three-fold. The presence of ROS, an early event in apoptosis, in AD1-8-tax cells incubated with paclitaxel, was indicated with the use of H_2DF-DA (Figure 7). AD1-8 cells treated the same way did not show the presence of ROS (data not shown). The TUNEL assay involves end-labelling of fragmented DNA with fluorescein-labelled nucleotides. This assay detected fragmentation of DNA in AD1-8-tax cells treated with paclitaxel, but not in AD1-8 cells (Figure 8).

Discussion

Recently, we were able to convert the non-paclitaxel binding tubulin in *S. cerevisiae* to a form that binds the drug efficiently by introducing five mutations into β-tubulin (Gupta *et al.*, 2003). To facilitate further studies of the effects of β-tubulin mutations on the binding efficiency of paclitaxel and its analogues, we desired a cell-based assay for the purpose of initial screening. Unfortunately, yeast cells that contain the paclitaxel-binding tubulin (MGY1-tax) are not sensitive to paclitaxel, presumably because of the multiple ABC transporters in yeast (Decottignies and Goffeau, 1997). Therefore, we introduced the mutated β-tubulin gene into a strain (AD1-8) that lacks the activities of seven of these transporters and one transporter transcription factor. Introduction of the mutated β-tubulin produced a strain (AD1-8-tax) that is sensitive to paclitaxel, albeit at relatively high concentrations compared to mammalian cells. The IC_{50} value for paclitaxel in mammalian cells is in the low nM range, about 0.1% of the value we found for *S. cerevisiae* strain AD1-8-tax. However, the 6 μM IC_{50} value is lower than we find for benomyl (~50 μM), a common anti-mitotic agent that inhibits yeast proliferation. Possibly, one or more of the ABC transporters remaining in the AD1-8-tax strain is capable of effluxing paclitaxel to a certain extent. It is also possible that the rather high IC_{50} value is due to the low sensitivity of *S. cerevisiae* proliferation to decreased microtubule dynamics. For example, we found that a β-tubulin C354S mutant proliferated at a rate equivalent to that of the wild-type strain, even though microtubule dynamics in the mutant were decreased by 73% to 98%, depending on the stage in the cell cycle (Gupta *et al.*, 2002). Thus, to affect cell proliferation, the paclitaxel concentration in the cell might have to be increased to such a level as to completely shut down microtubule dynamics.

Apoptosis, a natural process of programmed cell death that is required for the proper development of multicellular organisms, is induced by a number of stress-producing treatments, including anti-mitotic agents. Apoptosis has been studied in mammalian cells for several decades, but only recently has it been shown that apoptosis occurs in budding yeast (Madeo *et al.*, 1997, 2002). Programmed cell death in budding yeast is triggered by a diverse group of treatments or insults that include DNA damage (Burhans *et al.*, 2003), oxygen stress (Madeo *et al.*, 1999), NaCl (Wadskog *et al.*, 2004), cell ageing (Laun *et al.*, 2001; Fabrizio *et al.*, 2004; Herker *et al.*, 2004), acetic acid (Ludovico *et al.*, 2002), pheromones (Severin and Hyman, 2002; Pozniakovsky *et al.*, 2005) and viral killer toxins (Reiter *et al.*, 2005). To this list we can now add microtubule-stabilizing agents. It has been proposed that apoptosis in yeast can serve as a model system for the process in higher eukaryotes, especially because of the ease of genetic manipulations in yeast (Madeo *et al.*, 2002). Yeast can now be used as a model system for studying apoptosis induced by antimicrotubule agents. The strain AD1-8-tax can also serve another useful purpose. Until now, drugs capable of stabilizing yeast microtubules *in vivo* have been lacking, and all effective microtubule poisons function as depolymerizing agents (e.g. nocodazole, benomyl). With strain AD1-8-tax and the use of paclitaxel, it is now possible to study the effect of dampening microtubule dynamics on a variety of biological processes in *S. cerevisiae*.

Acknowledgments

This work was supported by NIH grant R01 CA105305 to R.H.H. We thank Dr. André Goffeau for supplying us with a culture of strain AD1-8.

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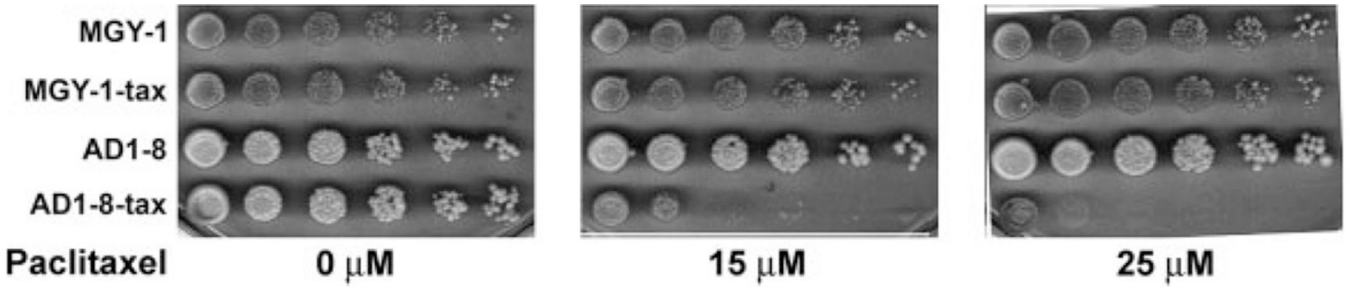

Figure 1.
Effect of paclitaxel on the growth of four yeast strains on agar plates. For experimental details, see Materials and methods

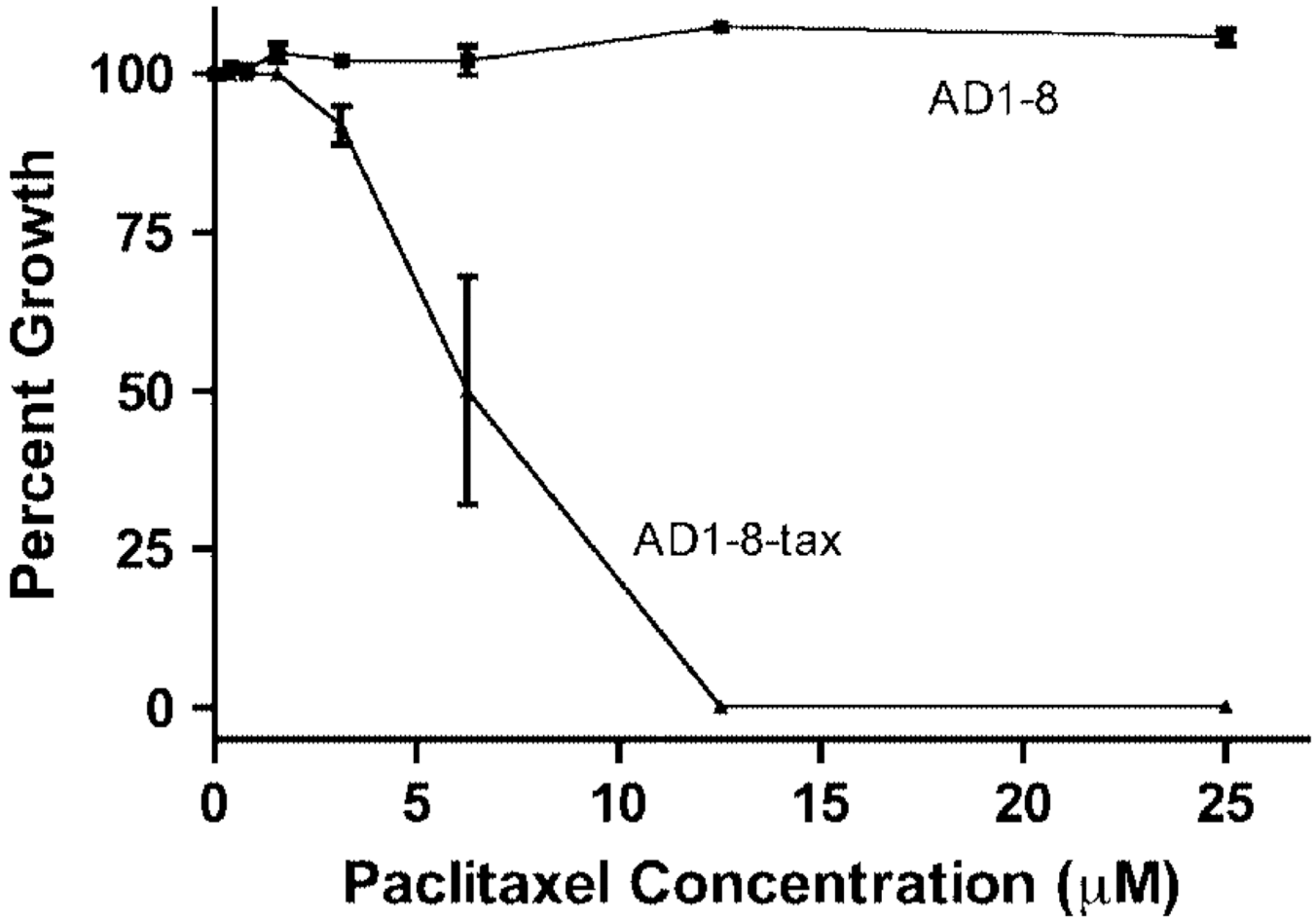

Figure 2.
Sensitivity of strains AD1-8 and AD1-8-tax to paclitaxel. Both strains were grown in liquid medium in the presence of increasing concentrations of paclitaxel, as described in Materials and methods. Percentage growth is shown relative to a culture without paclitaxel

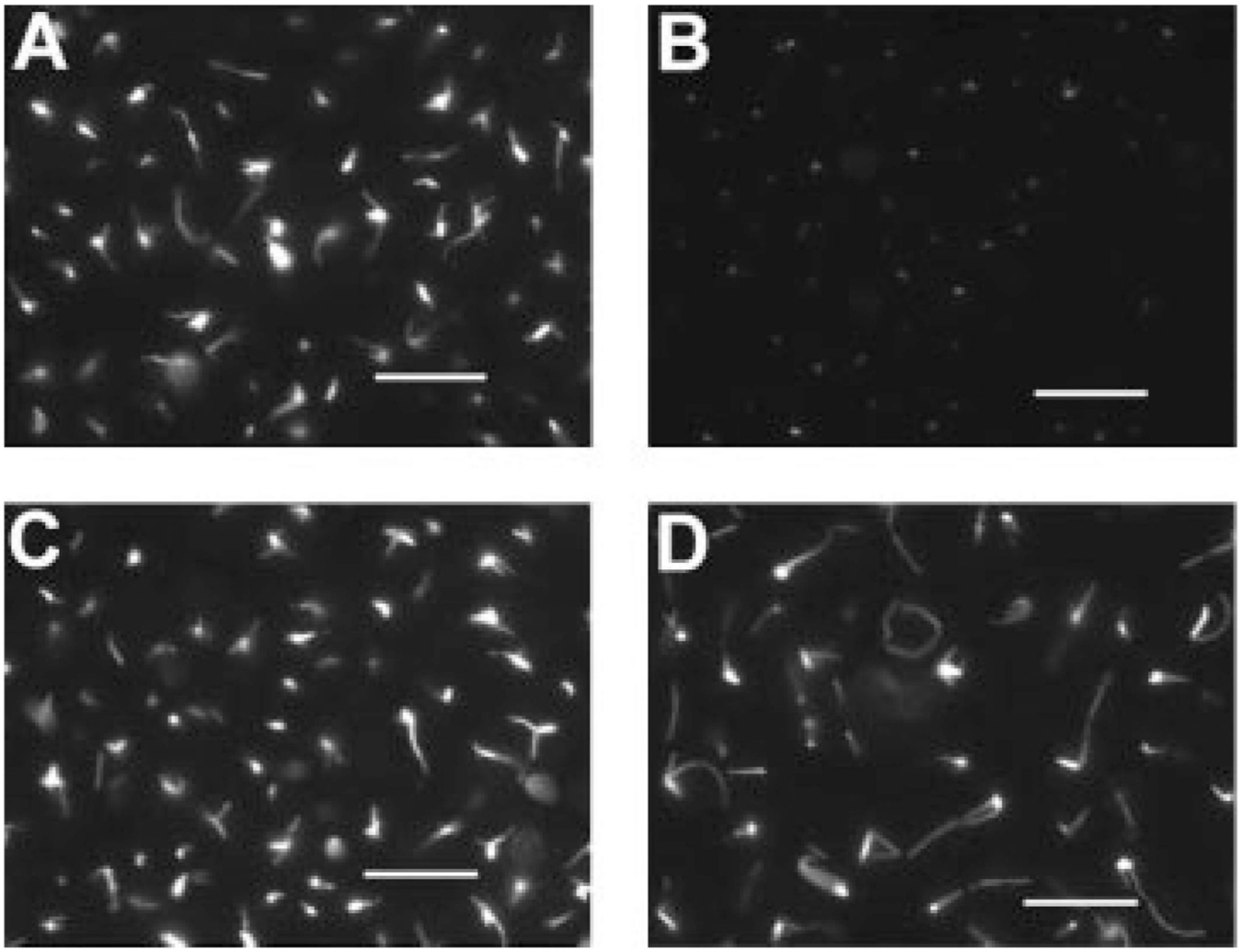

Figure 3.

Stability of microtubules in the presence of paclitaxel. Cultures of AD1-8 and AD1-8-tax were incubated with 25 μM paclitaxel at 30 °C for 6 h. Samples were taken for fixation and immunostaining. The remainder of the cultures were placed at 4 °C for 20 h before fixation and immunostaining. A and B, AD1-8 at 30 °C and 4°C, respectively; C and D, AD1-8-tax at 30 °C and 4°C, respectively. Bars = 10 μm

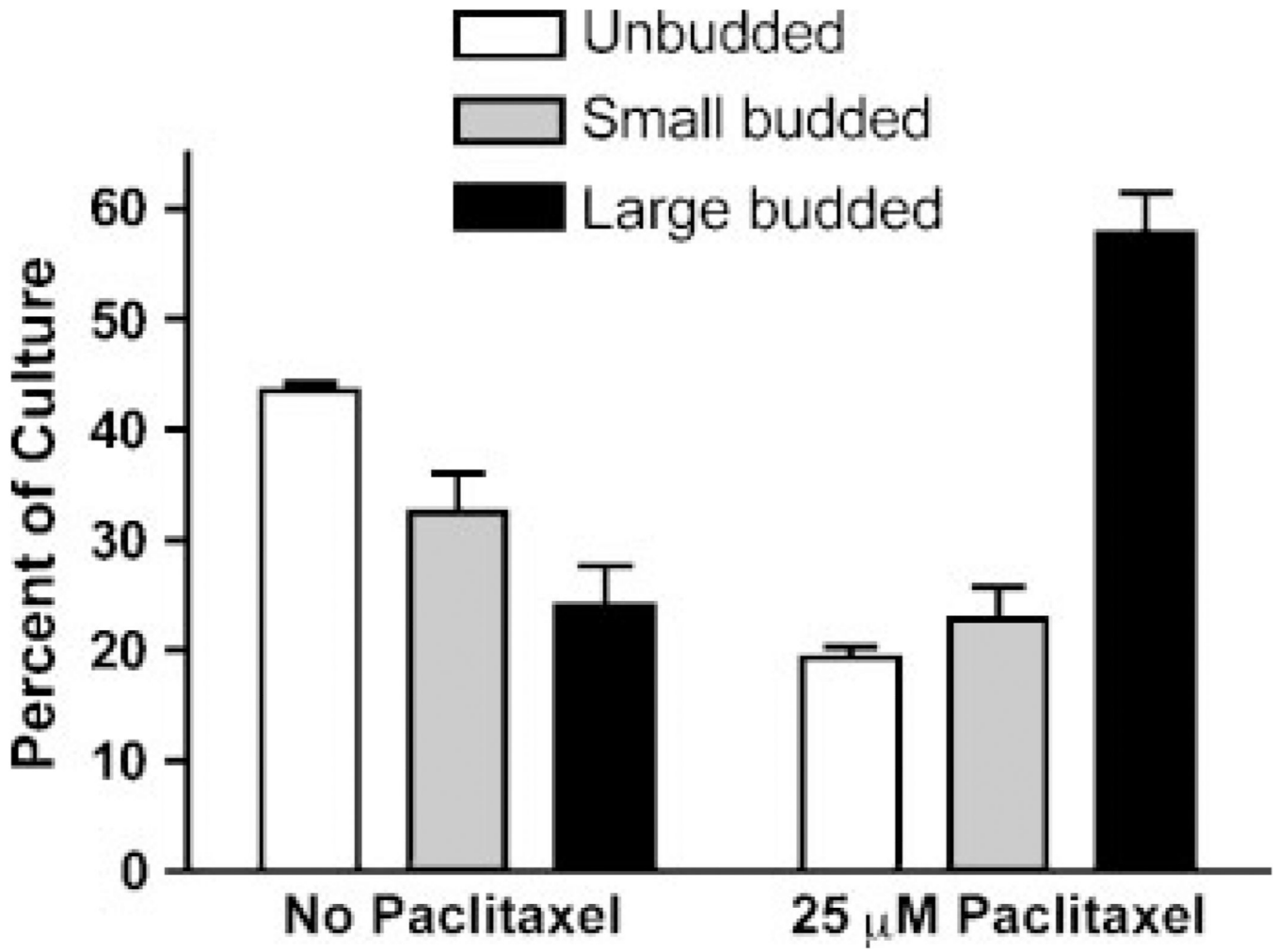

Figure 4.
Effect of paclitaxel on bud morphology in strain AD1-8-tax. Cultures were incubated in the absence and presence of 25 μM paclitaxel for 6 h before being examined for bud morphology. The results are from three separate experiments, with $n = 220$ in each case

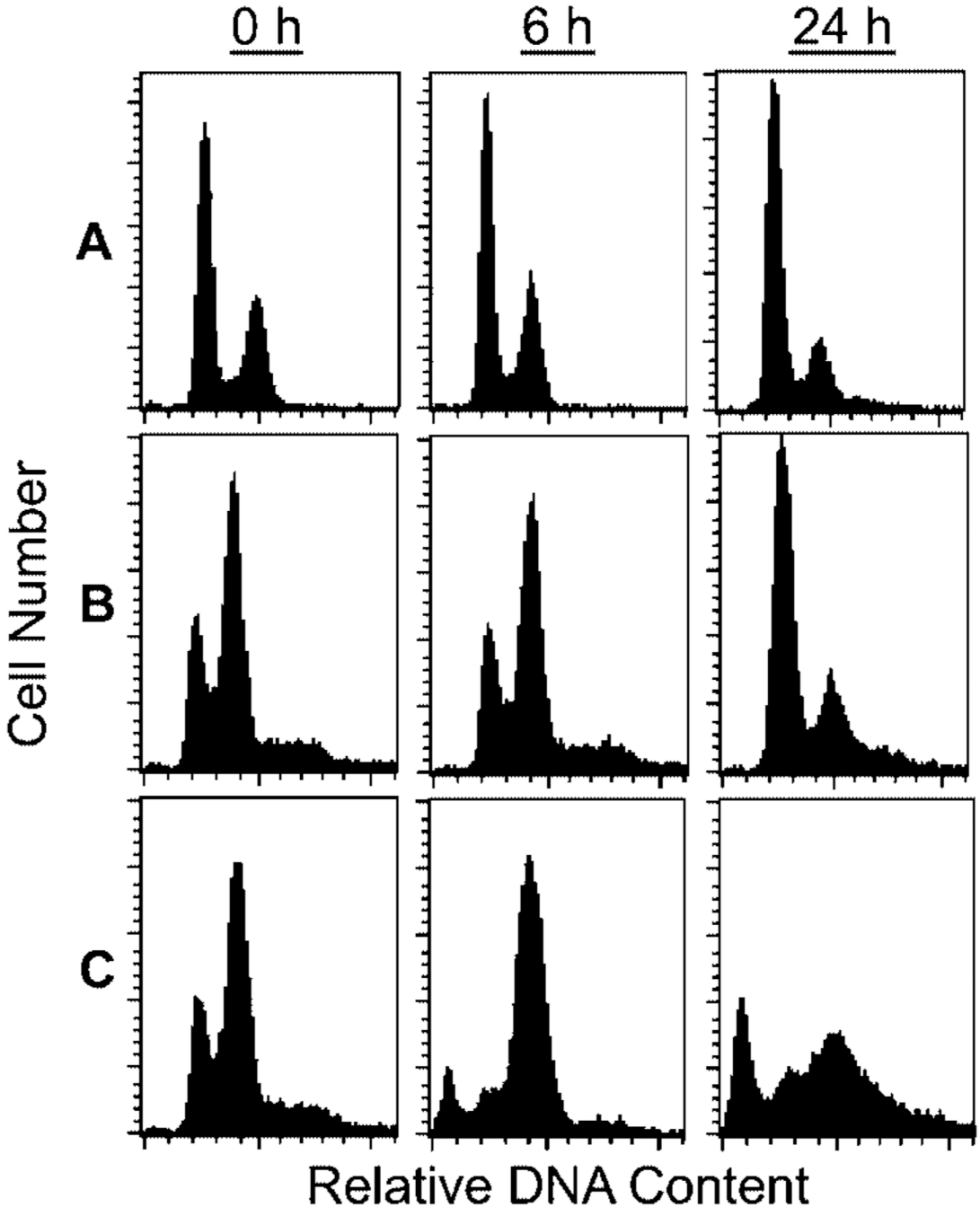

Figure 5.
FACS analysis of DNA content. Log phase cells were incubated with 25 μM paclitaxel for 6 h and 24 h before being prepared for FACS analysis, as described in Materials and methods. A, strain MGY1; B, strain AD1-8; C, strain AD1-8-tax

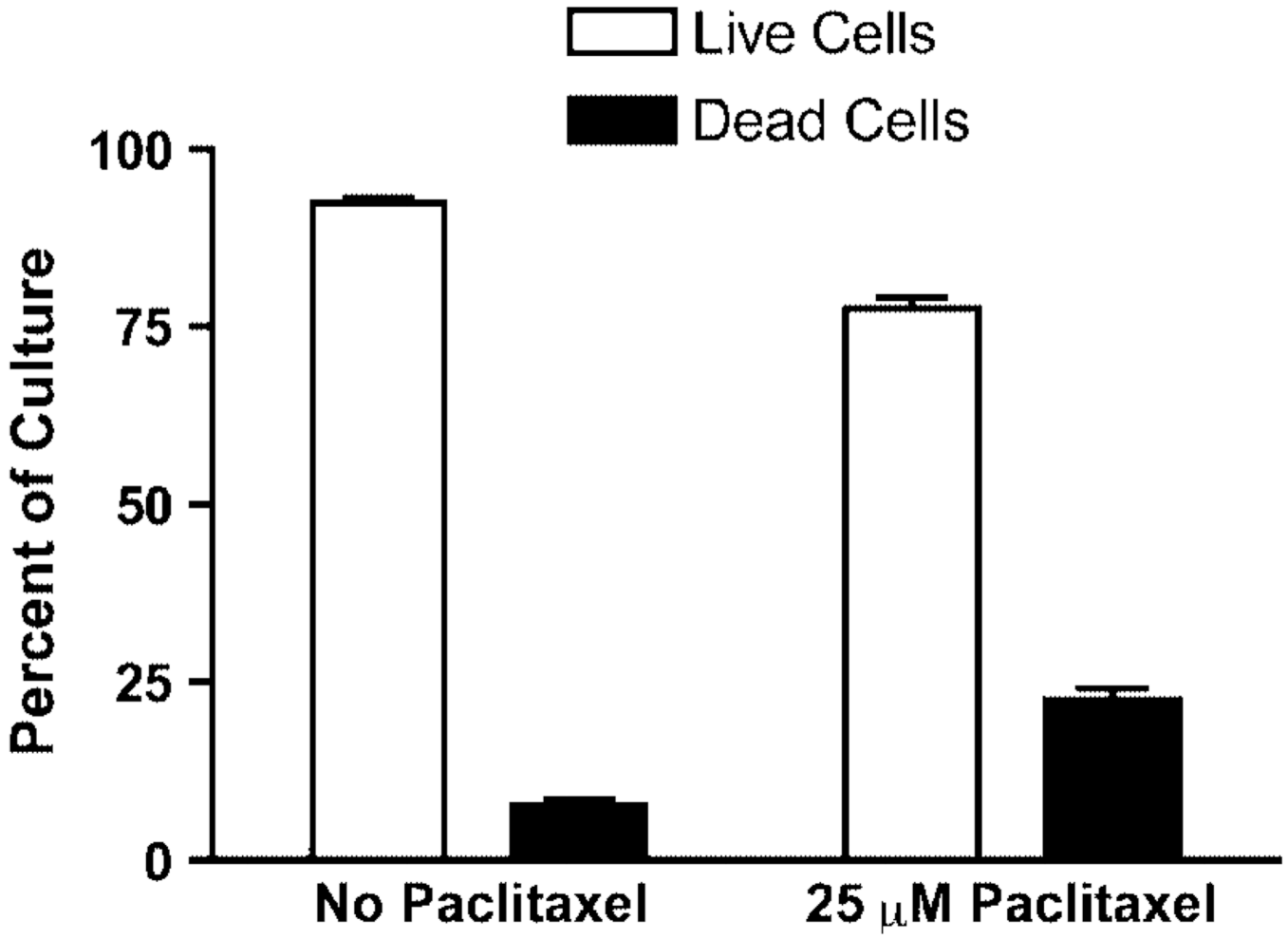

Figure 6.
Cell death produced by paclitaxel. Strain AD1-8-tax was incubated at 30 °C for 6 h in the absence and presence of 25 μM paclitaxel before being stained by phloxine B. The results are from three separate experiments, with $n = 200$ in each case

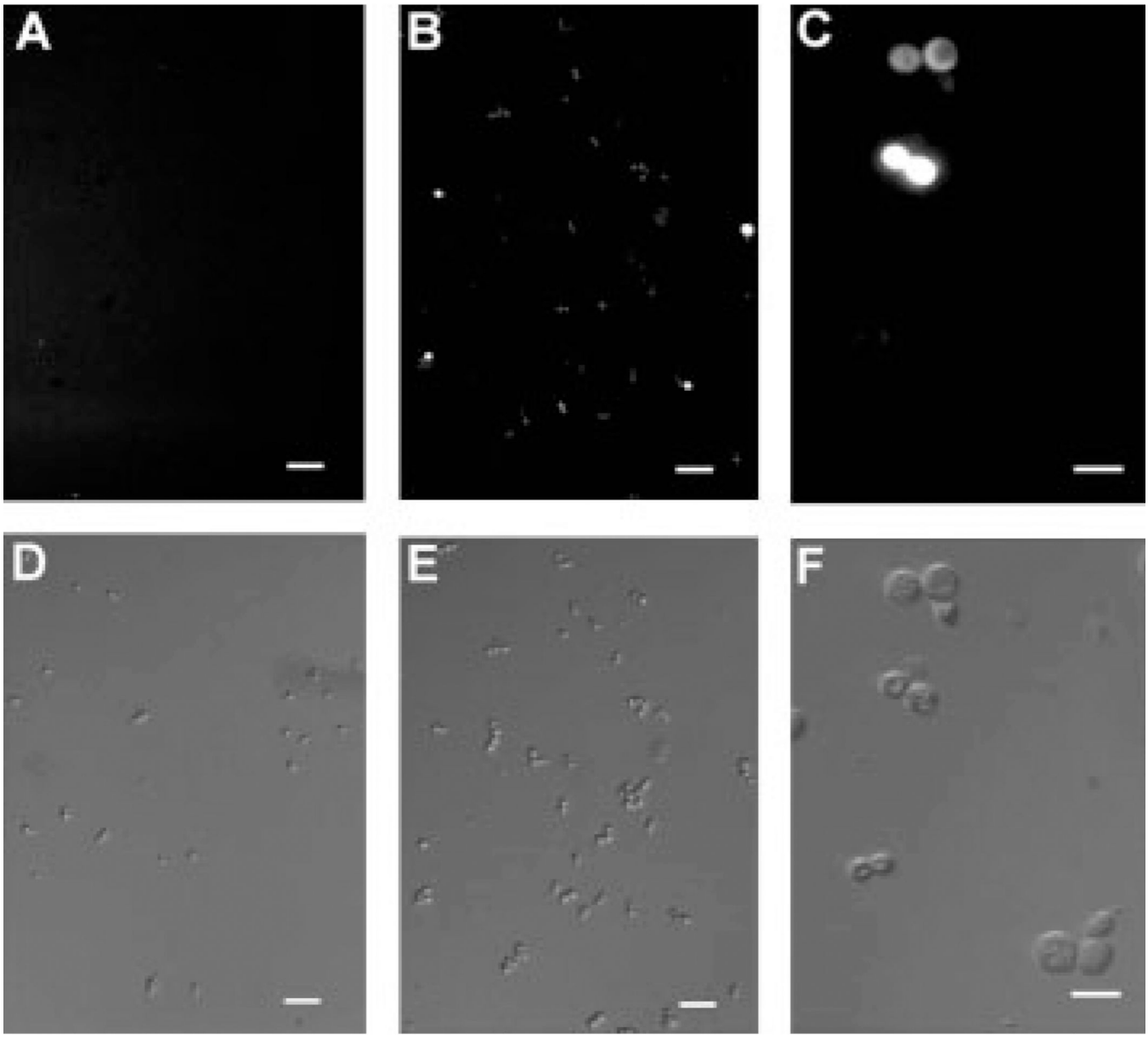

Figure 7.

ROS production after incubation with paclitaxel. Cultures of AD1-8-tax cells were incubated for 6 h at 30 °C in the absence (A, D) and presence (B, C, E, F) of 25 μM paclitaxel. DIC microscopy was used in (D–F.). ROS was detected with the compound H_2DF-DA using fluorescence microscopy (A–C). In (A, B, D, E) a 10× objective was used and bars = 30 μm; in (C, F) a 40× objective was used and bars = 10 μm

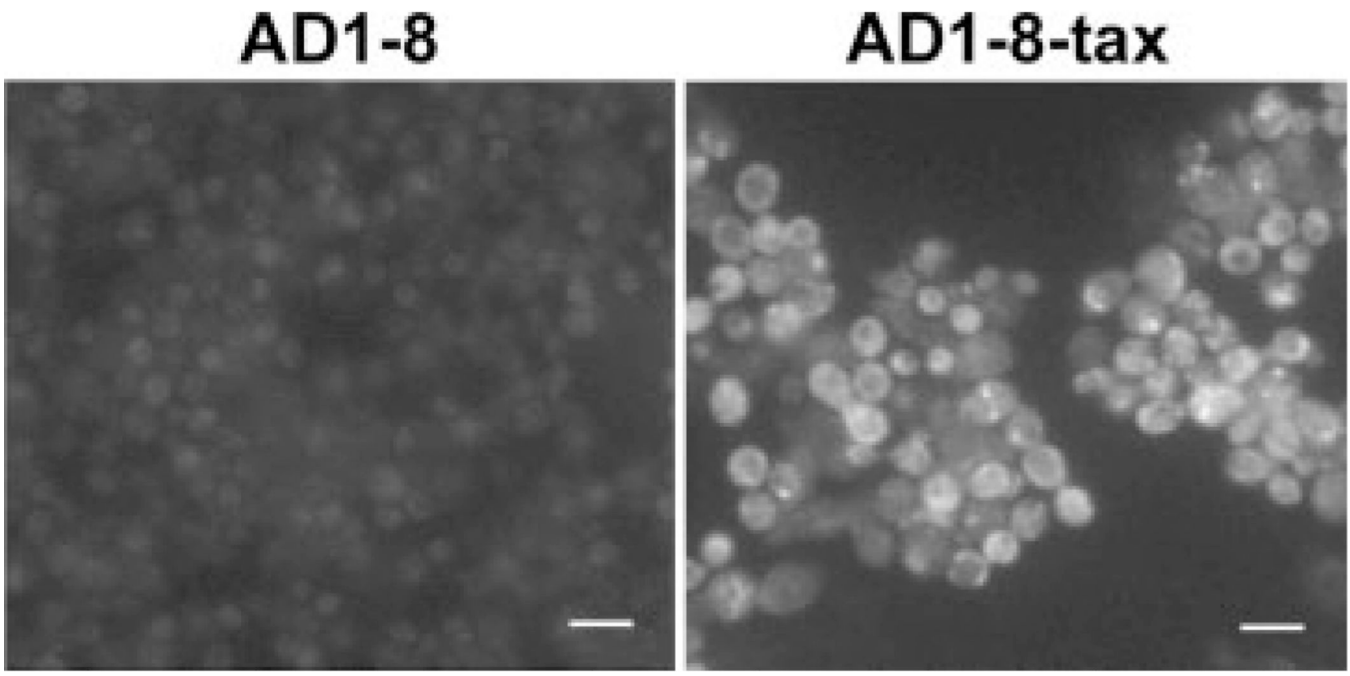

Figure 8.
Fragmented DNA determined by the TUNEL assay. Strains AD1-8 and AD1-8-taxwere incubated at 30 °C for 24 h in the presence of 25 μM paclitaxel. AD1-8-tax cells, but not AD1-8 cells, display fluorescence due to DNA labelled with fluorescein. Bars = 10 μm