

Characterization of the Hollow Fiber Assay for the Determination of Microtubule Disruption *In vivo*

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ABSTRACT

Purpose: The hollow fiber assay is used successfully as a routine *in vivo* screening model to quantitatively define anticancer activity by the National Cancer Institute. This study investigates whether the hollow fiber assay can be used as a short-term *in vivo* model to demonstrate specific pharmacodynamic end points, namely microtubule and cell cycle disruption.

Experimental Design: The growth of A549 cells was characterized within hollow fibers over 5 days *in vivo* at both subcutaneous (s.c.) and intraperitoneal (i.p.) sites. Drugs were administered on day 4 (i.p.).

Results: At 24 hours, cells were retrieved from fibers at both i.p. and s.c. sites of paclitaxel-treated (20 mg/kg) and combretastatin A1 phosphate-treated (150 mg/kg) mice. Cell cycle analysis after paclitaxel treatment revealed a mean G₂-M phase population of 48.04% (i.p.) and 25.76% (s.c.) compared with vehicle group mice (6.78 and 5.56%, respectively; $P = <0.001$ and 0.005, respectively). Tumor cells retrieved from combretastatin A1 phosphate-treated mice had a mean G₂-M phase population of 36.3% (i.p.) and 29.36% (s.c.) compared with cells retrieved from vehicle group mice (5.58 and 5.49%, respectively; $P = <0.001$). Using fluorescence and laser-confocal microscopy, paclitaxel was revealed to induce the formation of spindle asters and tubulin polymerization. Combretastatin A1 phosphate was shown to hold cells in mitosis. Changes in nuclear morphology were also observed.

Conclusion: These data demonstrate that the hollow fiber assay can be used as a short-term *in vivo* model for studying the pharmacodynamic effects of both standard and novel compounds on microtubules. Evidence has also been provided to support the routine use of the *in vivo* hollow fiber assay for demonstrating the mechanism of action of a drug.

INTRODUCTION

Based on previous microencapsulation and hollow fiber culture systems (1–4), Hollingshead *et al.* (5) developed the hollow fiber assay. In brief, this assay involves the short-term growth of tumor cells within biocompatible hollow fibers implanted at both the s.c. and i.p. sites of immunocompromised mice. The pharmacologic capacity of a compound to reach two physiologic compartments of the nude mouse is assessed along with the ability to demonstrate therapeutic efficacy at these sites. Three fibers are implanted at each site. One mouse can support the growth of up to six cell lines (5). The hollow fiber assay demonstrates a practical means of quantifying viable *in vivo* tumor cell mass through the use of a stable end point dye conversion technique and furthermore is not limited by the high costs associated with large-scale animal testing using xenograft tumor models.

Due to the feasibility of growing more than 50 tumor cell lines within hollow fibers *in vivo* and the relatively rapid and cost effective demonstration of *in vivo* activity by Hollingshead *et al.* (5) the National Cancer Institute (NCI) used the hollow fiber assay in 1995 as a routine preliminary *in vivo* screening assay. Presumably, this assay would prioritize compounds for secondary xenograft screening and help reduce the large number of active compounds generated by the *in vitro* 60-cell line screen that were forming a bottleneck for entry into secondary xenograft testing.

Since the development of the hollow fiber assay for drug screening, other groups have used this assay to independently demonstrate drug activity. Compound efficacy has been demonstrated using the hollow fiber assay both *in vitro* and *in vivo*, using both primary human tumor cells and characterized tumor cell lines and using various hosts. Antitumor activity has commonly been demonstrated using techniques that determine cell viability [*e.g.*, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay], as well as defining more specific states of cell death (*i.e.*, apoptosis and necrosis). Although it is recognized that the hollow fiber assay is implemented as a rapid drug screening model, no definitive mechanisms of drug action have been demonstrated by these studies (6–12).

The hollow fiber model potentially permits the retrieval of a pure population of tumor cells from within fibers. Other authors have taken advantage of this practical feasibility and have assessed tumor cell populations for more than cell viability status. Hollingshead *et al.* (13) were the first authors to look

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beyond cell survival assays as a measure of compound activity using the hollow fiber assay (13). Human lymphoid cells infected with the human immunodeficiency virus were retrieved from fibers after treatment with antiviral agents, and levels of p24 antigen and reverse transcriptase activity were measured. Several groups have subsequently retrieved cells from fibers after *in vivo* treatment and have subjected these to proteomic/genomic analysis (14–16). The implantation of hollow fibers has been demonstrated in immunocompetent (8, 9, 11, 17) and immunodeficient mouse strains (6, 7, 14–16) without causing immune destruction by the host or impeding the growth of tumor cells.

Currently, the hollow fiber assay is used routinely as a screening model for anticancer drug discovery. This assay does not define any precise mechanisms of action that may have previously been indicated by the recently molecularly characterized *in vitro* 60-cell line screen from which compounds have progressed. The present study aim was to provide evidence that the hollow fiber assay can be used as a short-term *in vivo* tumor model for the demonstration of predefined pharmacodynamic end points. The molecular target of interest in this instance is tubulin. The effects of the standard agent paclitaxel and the investigational agent combretastatin A1 phosphate on tubulin and the subsequent effects on cell cycle kinetics will be investigated using laser confocal microscopy and flow cytometry.

Paclitaxel (Taxol) has shown impressive activity in the treatment and palliation of several human solid tumor types, including ovarian, breast, lung, and head and neck cancers and was discovered as part of an NCI program in which extracts of some 30,000 plants were screened for anticancer activity. The actual natural source proved to be *Taxomyces andreanae*, a fungal endophyte isolated from the phloem of the pacific yew *Taxus brevifolia*. The antitumor efficacy of paclitaxel has been reported in several tumor models (18, 19).

Initially, it was indicated that paclitaxel could inhibit mitosis (20), and that paclitaxel could promote the assembly of stable bundles of microtubules from α - and β -tubulin heterodimers and inhibit their depolymerization back to tubulin (21). Later, the cellular target of paclitaxel was identified as tubulin. Paclitaxel was found to uniquely bind to the intermediate domain of the β -tubulin subunit (22, 23). Prominent morphologic features characteristic of paclitaxel-treated cells include the formation of microtubule bundling in interphase cells and spindle asters during mitosis (24). Formation of these stable bundles of microtubules disrupts the normal dynamics of the tubulin/microtubule system and results in the arrest of cells in G₂-M phase of the cell cycle (24). The consequent arrest of the cell cycle has been associated with the induction of apoptosis (25).

The combretastatins are a family of compounds derived from the african tree *Combretum caffrum* (26) that have antitumor and antivascular activity (27, 28). Combretastatin A1 phosphate is a more water-soluble analog of combretastatin A1 presently under preclinical investigation. Like its predecessor combretastatin A4, combretastatin A1 phosphate binds tubulin at or near the colchicine-binding site (29). Presumably, combretastatin A1 phosphate exerts its antitumor activity via an antivascular mechanism mediated by its tubulin-binding effects on the cytoskeleton of human tumor endothelial cells. More

recently, the greater antitumor effect of combretastatin A1 phosphate compared with combretastatin A4 phosphate (CA4P) has been proposed to be associated with differences in pharmacokinetics and metabolism (30).

In summary, this study was directed at providing evidence that the hollow fiber assay can be used as a short-term *in vivo* tumor model for the demonstration of predefined pharmacodynamic end points. Paclitaxel and combretastatin A1 phosphate, the most active of the combretastatin family to date, have been selected for study due to their common properties of tubulin binding.

MATERIALS AND METHODS

Cell Culture. All cell culture procedures were carried out in sterile microflow class II biological safety cabinets (MDH, Andover, United Kingdom). The A549 lung carcinoma cell line (31), previously confirmed as *Mycoplasma*-free, was cultured using Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich, St. Louis, MO) containing 10% fetal calf serum (FCS; Sigma-Aldrich), 1% penicillin-streptomycin (50 μ g/mL; Sigma-Aldrich), 1% L-glutamine (2 mmol/L; Sigma-Aldrich), and 1% sodium pyruvate (1 mmol/L; Sigma-Aldrich). Cells were grown in 75-cm³ flasks (Costar, High Wycombe, United Kingdom) in a humidified incubator at 37°C with 5% CO₂ (Heraeus Instruments, South Plainfield, NY). This human tumor cell line was chosen on the basis of showing sensitivity toward paclitaxel *in vitro* in preliminary studies.

Alternatively, A549 cells were grown on coverslips *in vitro* for use in microscopy studies. Sterile coverslips were placed in 6-well plates (Costar), and 5 mL of RPMI 1640 (10% FCS) were added to each well. Approximately 2×10^4 cells were added to each well and incubated in a humidified incubator at 37°C with 5% CO₂ for 3 to 4 days. After treatment regimens, coverslips were washed with Hanks' balanced salt solution and placed in methanol (100%) at -20°C for a minimum of 1 hour before immunostaining.

Hollow Fiber Preparation. All hollow fiber procedures were based on those of Hollingshead *et al.* (5). Polyvinylidene fluoride hollow fibers (type f; Spectrum Laboratories, Inc., Houston, TX) with a M_r 500,000 cutoff and 1-mm inner diameter were used. Tumor cells were cultured as described above, a subconfluent flask of cells attained and seeded at the desired density into hollow fibers. Fibers were placed in a 75-cm³ (Costar) flask of RPMI 1640 (20% FCS) and incubated at 37°C (5% CO₂) overnight.

***In vivo* Implantation of Hollow Fibers.** Pure strain female NMRI mice (B & K Universal, Ltd., Hull, United Kingdom) or NCR/Nu (National Cancer Institute, Frederick, MD) mice, ages 6 to 8 weeks, were used. All mice had access to food (CRM diet; SDS, Witham, Essex, United Kingdom) and water *ad libitum*. All animals were kept in cages in an air-conditioned room with alternating cycles of light and dark. All animal studies were carried out under a home office license. United Kingdom Co-ordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia were adhered to throughout the study (32). Mice were anesthetized by inhalation of isoflurane (Rhodia Organique Fine, Ltd). Three fibers were implanted at both subcutaneous (s.c.) and intraperi-

toneal (i.p.) sites. Incisions (i.p. and s.c.) were closed with histoacryl tissue glue (B/braun Surgical, Barcelona, Spain) with an additional skin staple.

Tumor Cell Retrieval from Hollow Fibers. Twenty-four hours after treatment *in vivo*, mice were sacrificed by cervical dislocation; and fibers excised, wiped to remove excess host tissue, and transferred to prewarmed RPMI 1640 (10% FCS) for 30 minutes. Fibers were transferred to 1 mL of prewarmed accutase solution (Innovative Cell Technologies; in Dulbecco's phosphate buffered saline 0.5 mmol/L EDTA) in 6-well plates. Fiber ends were cut and discarded. Fibers were flushed through once with the 1 mL of accutase solution. Plates were then incubated at 37°C (5%CO₂) for 30 to 45 minutes on an orbital shaker at 100 rpm (Lab-Line Instruments, Melrose Park, IL) to detach cells from fiber walls and attain a single cell suspension.

Assessment of Tumor Cell Growth within Hollow Fibers. On each day of growth, assessment mice were sacrificed by cervical dislocation, fibers were excised, and a modified version of the MTT dye conversion assay was performed (5). Absorbance was measured at 540 nm using a spectrophotometer (Multiscan Plus; Labsystems, Life Sciences International, Ltd., Basingstoke, United Kingdom; Genesis Labsystems software version 3.04). The percentage of net cell growth was calculated as follows:

$$\frac{\text{Mean absorbance on retrieval day} - \text{Mean absorbance on implantation day}}{\text{Mean absorbance on implantation day}} \times 100$$

Chemotherapy. Mice were treated on day 4 after hollow fiber implantation. Paclitaxel (M_r 853.9; Sigma-Aldrich) was dissolved in dimethyl sulfoxide:cremaphor:saline (1:1:8; 20 mg/kg). Combretastatin A1 phosphate (M_r 580.24) was synthesized at Arizona State University (33) and dissolved in sterile saline (150 mg/kg; ref. 34). Compounds were given 0.1 mL/10 g bodyweight by a single i.p. injection.

Mononuclear Cell Isolation. Mice were sacrificed using CO₂ gas. Approximately 0.5 mL of blood was withdrawn from the vena cava using a 21-gauge \times 1.5-inch needle, transferred to a heparinized tube to prevent coagulation, and placed on ice. Blood was carefully layered onto 1 mL of Ficoll-Paque (Amersham International Biosciences, Buckinghamshire, United Kingdom) in a 1.5-mL centrifuge tube and spun at 450 \times g (5417R centrifuge; Eppendorf, Hamburg, Germany) for 20 minutes at room temperature. The mononuclear layer at the interface between the two layers was transferred to a 1.5-mL centrifuge tube. Cells were resuspended in 1 mL of cold RPMI 1640 and centrifuged at 200 \times g for 5 minutes (4°C). Supernatant was removed, and 1.5 mL of ice cold 70% ethanol were added to the remaining cell pellet to fix cells. Cell suspensions were kept on ice for a minimum of 30 minutes and subjected to flow cytometry preparation methods.

Flow Cytometry. Fibers were excised from mice, and tumor cells were retrieved and prepared for flow cytometry analysis to assess progression through the cell cycle. Methods used were based on those of Ormerod (35). Analysis was performed using a Becton Dickinson flow cytometer using an argon-ion laser tuned to 488 nm and measuring forward and

orthogonal light scatter and red fluorescence. The percentage of cells in G₁, S, and G₂-M phases of the cell cycle were calculated using Sylchred and WinMDI software. Flow cytometry data were transferred to Microsoft Excel spreadsheets and analyzed for statistical significance using the Student's *t* test—two sample.

A preliminary experiment was undertaken to determine the minimum cell numbers required to produce a normal DNA histogram. Cell densities greater than 5 \times 10⁴ cells/mL were adequate for generation of DNA histograms from which data could be successfully analyzed (data not shown).

Immunofluorescence Microscopy. Before cytospinning cells onto slides, a preliminary study was undertaken whereby tumor cells were cytospun at variable speeds and durations to determine optimum conditions that would limit disruption of microtubules. Tumor cells were retrieved from hollow fibers and pelleted, cold PBS (200 μ L) was added, and cells were placed on ice, cytocentrifuged at 800 \times g (Shandon 4; Shandon, Inc., Pittsburgh, PA; 1 minute) onto glass slides, and placed immediately in 100% methanol (−20°C) for a minimum of 1 hour to fix cells. Slides were washed in PBS for 10 minutes. Primary antibody anti- α -tubulin clone DM1A (1:500; Sigma-Aldrich; 150 μ L) was added to each slide and incubated for 30 minutes. Slides were washed in PBS (three times for 5 minutes). Immunostaining procedures from this point were carried out in the dark. Secondary antibody rabbit antimouse immunoglobulin tetramethylrhodamine isothiocyanate (1:50; 150 μ L; Dako) was centrifuged at 15,000 \times g (5 minutes; MIKRO 20; Hettich Zentrifugen, Tuttlingen, Germany) and added to slides for 30 minutes. Slides were washed in PBS (three times for 5 minutes). Hoechst stain 33342 (1:100; Sigma-Aldrich) was added to slides and incubated for 30 minutes. Slides were washed in PBS (10 minutes), mounted in antifading medium (Dako fluorescence mounting medium), and stored at 4°C in the dark. Images of microtubule morphology were taken using a confocal microscope (Bio-Rad Microradiance Confocal Imaging System attached to a Nikon CM-800 microscope using Laser Sharp 2000 software; excitation and emission maxima, 435 and 570 nm, respectively).

RESULTS

Characterization of Tumor Cell Growth within Hollow Fibers *In vivo*. The importance of characterizing the initial cell loading density of hollow fibers with respect to enhancing compound efficacy has been demonstrated previously (14). Tumor cell growth within hollow fibers was characterized so that exponential growth could be defined. During the linear phase of growth, there are a greater proportion of cells dividing, which potentially makes the tumor cell population more sensitive to agents whose molecular target is tubulin. A549 cells seeded at 1 \times 10⁶ cells/mL were defined as the optimal seeding density for *in vivo* growth within hollow fibers over 5 days at both i.p. and s.c. sites (Fig. 1). Additional *in vivo* hollow fiber studies involving paclitaxel and combretastatin A1 phosphate treatment were performed using this cell seeding density.

Flow Cytometry. NMRI mice were treated with the standard tubulin-binding agent paclitaxel (20 mg/kg; i.p.). At 24 hours, fibers were excised, and cells were retrieved and prepared for flow cytometry analysis. Cell numbers retrieved from each

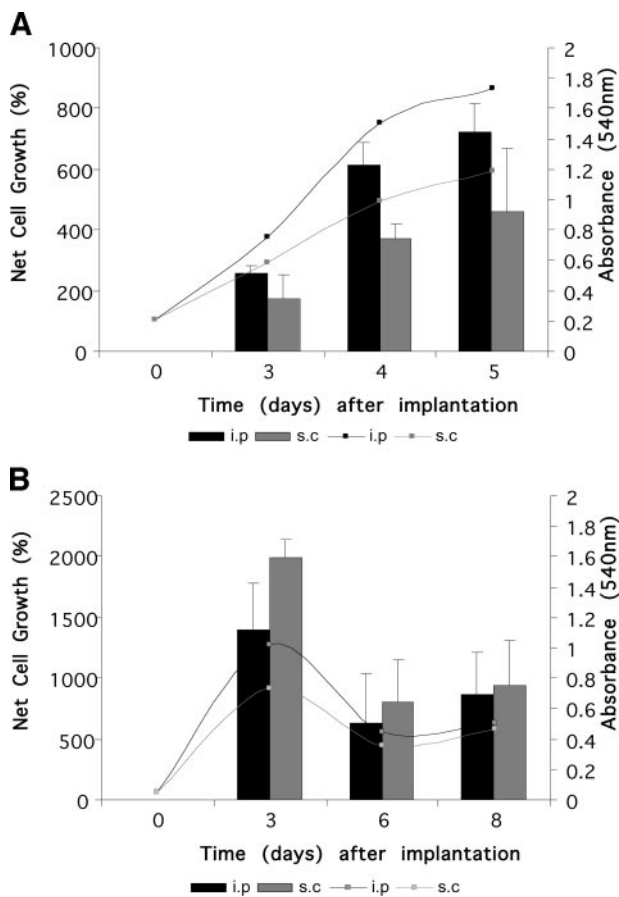


Fig. 1 Graph to show the net cell growth (%) and absorbance values (540 nm) of A549 cells grown in hollow fibers *in vivo* seeded at 1×10^6 cells/mL (A) and 2.5×10^6 cells/mL (B). Fibers were incubated overnight (37°C), implanted into NMRI mice, and retrieved at each time point. Results are expressed as mean data from nine fibers (three per mouse per site) \pm SD. Net cell growth (%) has been presented (■, i.p.; ▒, s.c.), which was calculated from absorbance values (black line, i.p.; gray line, s.c.) generated using the MTT assay.

hollow fiber were sufficient to produce a DNA histogram. DNA histograms produced from each experiment were used to generate mean data (Fig. 2). Results revealed cells retrieved from fibers from paclitaxel-treated mice to have a mean G₂-M phase population of 48.04% (i.p.) and 25.76% (s.c.) compared with vehicle groups (6.78 and 5.56%, respectively; **, $P < 0.001$; *, $P < 0.005$; Fig. 2A). Similarly NMRI mice were treated with the investigational tubulin-binding agent combretastatin A1 phosphate (150 mg/kg; i.p.). At 24 hours, fibers were excised, and cells retrieved and prepared for flow cytometry analysis. A549 cells retrieved from fibers from combretastatin A1 phosphate-treated mice were shown to have a mean G₂-M phase population of 36.3% (i.p.) and 29.4% (s.c.) compared with control groups (5.6 and 5.5%, respectively; **, $P < 0.001$; Fig. 2B).

A conspicuous pre-G₁ peak was sometimes present in DNA histograms in both treatment and vehicle groups. The nature of this pre-G₁ peak was investigated, and it was determined to be host cell material (Fig. 3). DNA histograms reveal the peak produced by NMRI mouse mononuclear cells to be identical to the pre-G₁ peak

produced by tumor cell samples retrieved from hollow fibers *in vivo*. Because no host cell migration into fibers has been observed by histologic assessment here or by any other groups, it is likely that this host cell contamination was a consequence of not being careful enough to remove all host material from the outside of fibers. Initially, it was considered that host cell contamination may also be a consequence of using an immunocompetent mouse strain. Additional studies revealed that this was not the case, because this pre-G₁ peak was still evident in tumor cell samples retrieved from nude mice (data not shown). Therefore it should be noted that host cells should be adequately removed from fibers to prevent contamination of tumor cell samples. The possibility of host cell contamination has been observed here due to the use of flow cytometry methods that are capable of identifying cells originating from different species, based on DNA diploid cell numbers. In other instances whereby tumor cells have been removed from fibers and

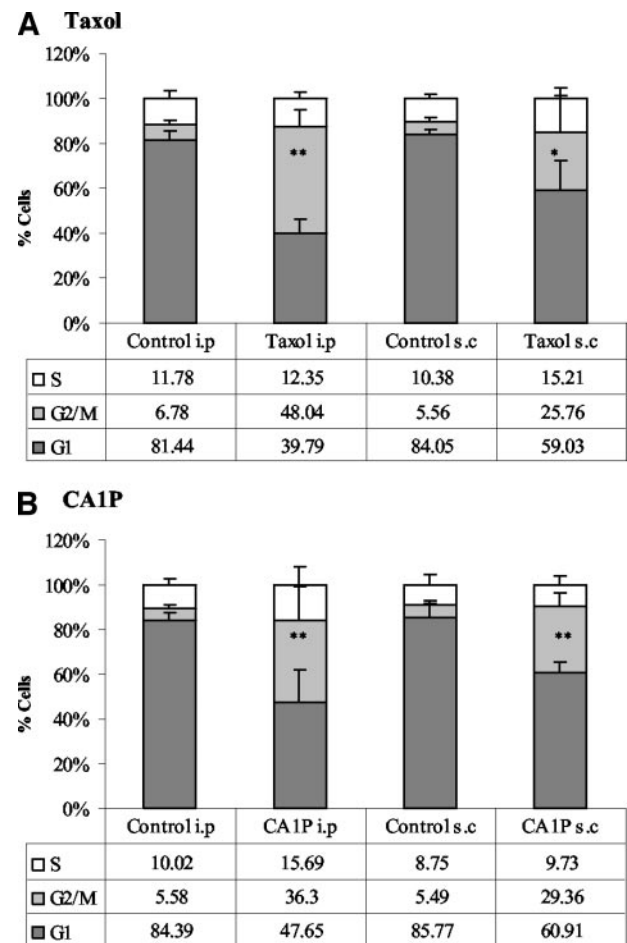


Fig. 2 Flow cytometry analysis of A549 cells seeded in hollow fibers at 1×10^6 cells/mL, incubated overnight, and implanted into NMRI at both s.c. and i.p. sites (three fibers per site). On day 4, mice were treated with either paclitaxel (A; 20 mg/kg) or combretastatin A1 phosphate (B; 150 mg/kg) or with a vehicle by a single i.p. injection. At 24 hours, fibers were excised, and cells were retrieved and subjected to flow cytometry analysis. Each block represents the mean of nine fibers retrieved from three mice (three fibers per site per mouse). Error bars, \pm SD.

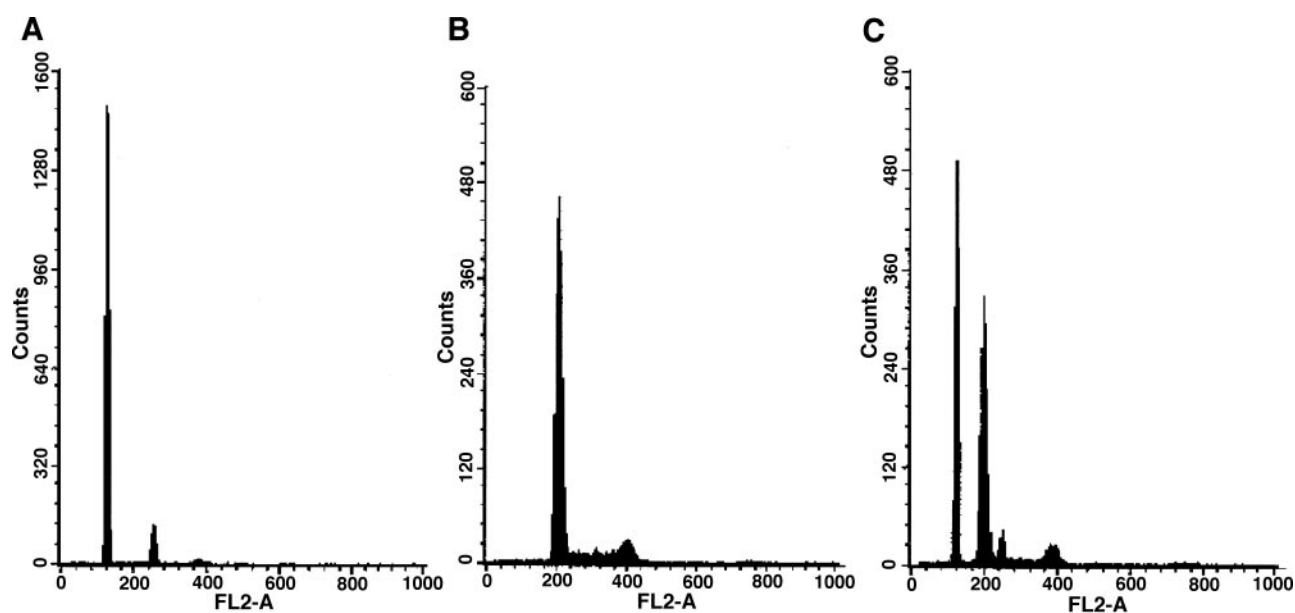


Fig. 3 DNA histograms of lymphocytes derived from NMRI mice (A); A549 cells (B); and both lymphocytes derived from NMRI mice and A549 cells (C).

subjected to molecular analysis for the determination of gene, RNA, and cell cycle marker expression (14–16), host cell contamination would not be recognized so easily.

Assessment of Cellular Morphology. With a view to demonstrating the pharmacodynamic effects of tubulin-binding agents on the microtubules of tumor cells in hollow fibers *in vivo*, initial studies were carried out *in vitro*. Any effects shown *in vitro* would be used as a reference point for observing pharmacodynamic effects in the *in vivo* hollow fiber assay, although it is appreciated that growing cells on coverslips provides an ideal snapshot of any potential drug effects. The quality of images produced from cells retrieved *in vivo* are slightly impaired by cytocentrifugation necessary for the examination of tumor cells retrieved from hollow fibers. Cytocentrifugation is disruptive to cellular microtubules, although the effects of tubulin-binding agents are still apparent. A549 cells were treated with clinically relevant concentrations of paclitaxel or combretastatin A1 phosphate *in vitro* to observe the effects of these compounds on the morphology of cellular microtubules. After treatment with paclitaxel *in vitro*, the prevalence of multiple mitotic asters and parallel arrays of microtubules were observed (data not shown). The first appearance of mitotic asters was observed at 1 hour (1 $\mu\text{mol/L}$). Loss of normal microtubule structure was evident after treatment with combretastatin A1 phosphate (10 $\mu\text{mol/L}$, 0.5 hour; data not shown). Having demonstrated the effects of both paclitaxel and combretastatin A1 phosphate against A549 lung carcinoma cells on cellular microtubules *in vitro*, we administered these compounds to mice bearing hollow fibers. Tumor cells were retrieved from hollow fibers 24 hours after treatment *in vivo*, cytospun, immunostained, and observed. Paclitaxel was shown to induce polymerization of normal microtubule networks. Mitotic asters were also evident. Populations of cells retrieved from fibers excised from combretastatin A1 phosphate-treated mice were shown to have a greater proportion of cells held

in mitosis and possessed cells lacking the usual detailed microtubule network structure (Fig. 4). Tumor cells were also stained with Hoechst 33342 so that nuclear morphology could be observed (Fig. 5). With treatment of both paclitaxel and combretastatin A1 phosphate, the normal uniform circular morphology of A549 cell nuclei was disrupted, and fragmented nuclei prevailed at 24 hours.

DISCUSSION

This study has successfully characterized the hollow fiber assay for investigating pharmacodynamic end points *in vivo*, namely microtubule disruption and consequent cell cycle disruption. These pharmacodynamic end points have been demonstrated *in vivo* using both the standard tubulin-binding agent paclitaxel and the investigational agent combretastatin A1 phosphate for the first time. Growth of A549 tumor cells within hollow fibers *in vivo* was initially characterized to determine the greatest window of sensitivity (linear growth). Optimization of cell seeding density and cell retrieval from hollow fibers permitted the retrieval of sufficient cell numbers for flow cytometry analysis from just one hollow fiber. Six DNA histograms were generated from one mouse (three fibers per site). These data demonstrate the use of the hollow fiber assay as a more cost-effective *in vivo* model than the xenograft model (5). For instance, cells may be retrieved from each fiber and generate three individual statistically relevant samples, whereas the use of the xenograft tumor model for such pharmacodynamic analysis is less feasible.

Paclitaxel has previously been used in an *in vitro* hollow fiber model (10). Assessment of antitumor activity was quantitative but did not indicate any precise mechanism of action. Here, paclitaxel has been used for the first time to demonstrate microtubule disruption using the *in vivo* hollow fiber model.

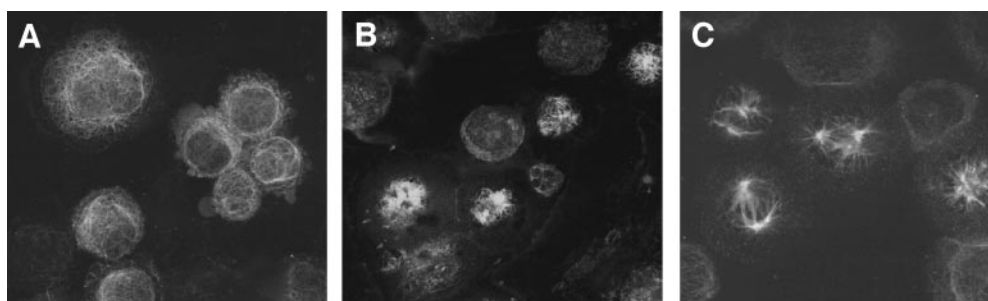


Fig. 4 A549 cells retrieved from hollow fibers implanted in NMRI mice, cytospun on to slides, and immunostained for α -tubulin at 24 hours after treatment. **A**, A549 cells retrieved from vehicle treated mice. Microtubules appear as fibrous networks. **B**, A549 cells retrieved from fibers excised from paclitaxel-treated mice. Cells appear as bundles of polymerized microtubules with mitotic asters visible. **C**, A549 cells retrieved from fibers excised from combretastatin A1 phosphate-treated mice. A relatively large number of cells appear to be undergoing mitosis and lack detailed microtubular networks.

Cell cycle analysis revealed a significant G₂-M block in tumor cells retrieved from hollow fibers excised from paclitaxel-treated mice. This G₂-M block is consistent with cell cycle arrest previously demonstrated *in vitro* upon treatment with paclitaxel (24, 25). Drugs that interfere with tubulin, and therefore with cell division, are known to cause this effect when analyzed using flow cytometry (36).

Supportive evidence of the observed cell cycle arrest in tumor cells retrieved from hollow fibers excised from paclitaxel-treated mice was derived from laser confocal microscopy studies. *In vitro* studies revealed the disruption of the normal microtubule cytoskeleton upon treatment with paclitaxel. Specific characteristics include the prevalence of abnormal bundling of microtubules, bundling in radial patterns of microtubules, formation of multiple mitotic asters, and parallel arrays of polymerized tubulin. The most visible effect of paclitaxel on cells is the formation of microtubule bundling in interphase cells and spindle asters during mitosis. These paclitaxel-induced morphologic characteristics are consistent with those previously documented *in vitro* (24). Despite cyto centrifugation of tumor cells causing some disruption to cellular microtubules compared with ideal conditions exhibited *in vitro*, the presence of multiple asters is still clearly observed after retrieval of tumor cells from *in vivo* hollow fiber studies. Horwitz *et al.* (37) have previously demonstrated microtubule bundle formation as a pharmacodynamic end point in a phase I clinical study using both peripheral blood mononuclear cells and breast tumor cells from patient

biopsies. This study used the more water-soluble epothilone analog BMS-247550, a competitive inhibitor of paclitaxel. Here, tubulin polymerization has been demonstrated as an indicator of drug-target interaction using paclitaxel in a preclinical *in vivo* model for the first time.

Tumor cells retrieved from hollow fibers excised from paclitaxel-treated mice revealed extensive fragmented nuclei in contrast to the uniform circular nuclear shape observed in tumor cells retrieved from vehicle-treated mice. This DNA degradation into nucleosome-sized fragments is characteristic of apoptosis (38) and has previously been observed in paclitaxel-treated cells (24, 25). Although there is compelling evidence that tubulin causes the stabilization of microtubule dynamics and consequent mitotic arrest leading to apoptosis, biochemical events downstream of tubulin binding and mitotic arrest are less clear (39).

Combretastatins are known to induce antitumor activity mediated by an antivascular mechanism. This has previously been demonstrated using both s.c. and orthotopic-metastasis models using combretastatin A1 phosphate (28, 34).

Combretastatin A1 phosphate has been shown here to induce tubulin depolymerization in tumor cells *in vitro*. Although combretastatin A1 has previously demonstrated inhibition of tubulin assembly *in vitro* (26), here for the first time, the combretastatin A1 prodrug (combretastatin A1 phosphate) has been shown to induce microtubule disruption in tumor cells both *in vitro* and *in vivo*.

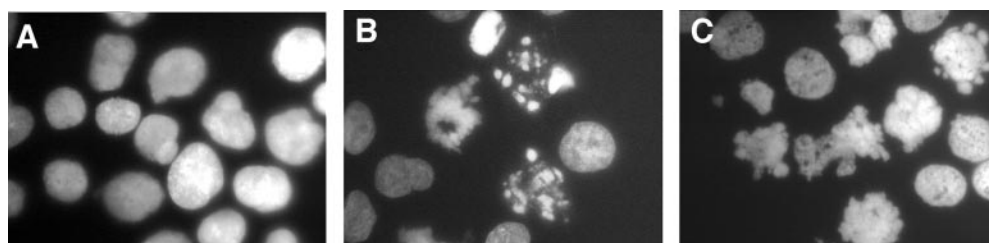


Fig. 5 A549 cells retrieved from hollow fibers implanted in NMRI mice were cytospun onto slides and stained with Hoechst 33342, 24 hours after treatment. **A**, A549 cells retrieved from vehicle treated mice, revealing normal uniform circular nuclei. **B**, A549 cells retrieved from fibers excised from paclitaxel-treated mice. Many cells possess irregular morphology and fragmented nuclei. **C**, A549 cells retrieved from fibers excised from combretastatin A1 phosphate-treated mice. Many cells possess fragmented nuclei.

This disruption of microtubule morphology was similar to that demonstrated *in vitro* using colchicine and CA4P (data not shown). Combretastatin A1 phosphate and its predecessor CA4P are known to bind tubulin at the colchicine-binding site and inhibit microtubule assembly (29). Apparently, the trimethoxybenzene moiety observed in colchicine and combretastatin probably represents a favored binding structure for tubulin (29). The importance of the *cis*-configuration over the *trans*-configuration has also been reported (40).

The hollow fiber assay was used to investigate the pharmacodynamic activity of combretastatin A1 phosphate *in vivo*. Flow cytometry analysis revealed a statistically significant G₂-M block in tumor cells retrieved from fibers excised from both s.c. and i.p. sites of NMRI mice. Here, it is likely that this combretastatin A1 phosphate-induced G₂-M block occurs due to the direct tubulin binding of combretastatin A1 phosphate as opposed to an antivascular mechanism as hollow fibers do not possess a well-developed surrounding vasculature at 5 days (17). Drug delivery is likely to be by extravascular diffusion. Recently, small molecule drug delivery to s.c. fibers has been demonstrated with the use of imaging technology (41).

Here, combretastatin A1 phosphate has been used for the first time to demonstrate cell cycle disruption *in vivo*. Previously, cell cycle arrest has been demonstrated in tumor cells using combretastatin A4 *in vitro* (36). Supportive evidence of this G₂-M arrest in tumor cells retrieved from fibers excised from mice treated with combretastatin A1 phosphate is derived from laser confocal microscopy studies whereby cells were found predominantly to be held in mitosis and lacked intricate networks of microtubules. Tumor cells retrieved from hollow fibers excised from combretastatin A1 phosphate-treated mice revealed multiple and fragmented nuclei and nuclear blebbing in contrast to the uniform circular nuclei shape observed in tumor cells retrieved from vehicle-treated mice. Such nuclear morphology is characteristic of apoptosis (38). Although apoptosis is thought to follow mitotic arrest with tubulin-binding agents, this has not until now been indicated with combretastatin A1 phosphate. Previously, nuclear morphology of tumor cells has been investigated with CA4P *in vitro*. The formation of giant, multinucleated cells characteristic of early mitotic catastrophe was observed, along with a G₂-M block. Only small numbers of apoptotic cells were detected (42). Additional studies are required to confirm apoptosis with the use of other methods (*e.g.*, flow cytometry, terminal deoxynucleotidyltransferase-mediated nick end labeling).

Currently, it is believed that *in vivo* models should be fully characterized to ensure that the precise target of interest is being expressed, rather than using mouse models for random screening (43). Furthermore, it has been suggested that characterized *in vivo* models that are more biologically representative of patient tumors are more predictive of clinical response than *in vivo* models that are not characterized (44). Human tumor xenograft models used in secondary *in vivo* drug screening are often poorly characterized. This lack of molecular characterization in xenograft models may contribute to the lack of success of compounds entering the clinic. Tumor cell lines from which the routine NCI xenograft panel are derived are representative of the main histologic types of cancer and have been molecularly characterized, but whether these specific molecular characteris-

tics are retained *in vivo* has not been confirmed. Any preclinical tumor response may not therefore be predictive of clinical activity.

Molecular drug targets such as VEGF/EGF/PDGF/FLT3 receptors, and the Bcr-Abl tyrosine kinase are becoming increasingly attractive targets. Small molecules designed to interact with these targets are emerging into the clinic. Although these molecular targets have been implicated in the progression of human malignancy, some recent clinical trials using small molecule inhibitors have yielded only modest response rates. It has been proposed that this is partially due to these drugs being administered to unselected patient populations, without precise knowledge of the molecular characteristics prevalent in each patient tumor (45).

The hollow fiber model may offer an attractive, relatively rapid and cost-effective *in vivo* model in which to culture patient tumor cells and moreover demonstrate drug interactions with clinically relevant molecular targets. Such a “personalized” treatment approach may increase overall clinical efficacy.

Recently, genetically engineered mouse models have been used to test the therapeutic efficacy of small molecule inhibitors (*i.e.*, FLT3 receptor tyrosine kinase). In such genetically engineered mouse models, the molecular target was characterized *in vivo* by transducing cells with activated FLT3 alleles (46). Preclinical efficacy demonstrated in these genetically engineered mouse models suggests that these agents will prove useful in treating acute myeloid leukemia in the clinic, especially considering that this model of murine leukemia is driven by mutations that correspond to those that cause disease in humans (47). The use of genetically engineered mouse/transgenic/knockout models for screening rationally designed small molecule inhibitors is limited by excessive costs incurred and time compared with classical xenograft models and moreover the hollow fiber assay. Perhaps the hollow fiber assay offers a more rapid, inexpensive *in vivo* model to study such molecular targets. Molecular analysis of signaling molecules downstream of the targeted receptor tyrosine kinase would be facilitated by the technical feasibility of tumor cell retrieval and would potentially indicate any upstream drug/target interaction.

Whether characterized tumor cell lines or primary cells are cultured, the hollow fiber model fulfils its role in determining the pharmacologic capacity and consequent therapeutic efficacy of a compound. The demonstration of the mechanism of action of a compound *in vivo* after administration at its maximum tolerated dose using the hollow fiber model questions whether the considerably more expensive, less ethical (number of animals required), and time consuming xenograft model is entirely necessary. Activity in xenograft models is defined by a reduction in relative tumor volume. With respect to today’s rationally designed small molecules, activity in the xenograft model does not easily permit elucidation of the mechanism of a drug. Tumor cell retrieval from xenograft models for pharmacodynamic analysis is complicated by host cell contamination. If the hollow fiber model was used for such preclinical pharmacodynamic investigations and as a replacement for routine xenograft screening, compounds may progress to the clinic much faster. Agents may also prove more effective if patients were selected for treatment on the basis of molecular suitability for the compound in question.

The *in vivo* hollow fiber assay has demonstrated good predictivity of xenograft activity, and therefore any compound found to be active in the xenograft model is unlikely to be missed by the hollow fiber assay (14, 48, 49). Upon identification and validation of new molecular drug targets implicated in the initiation and progression of human malignancy, the appropriate design of the hollow fiber assay and molecular characterization of tumor cells may allow confirmation of clinically relevant drug and/or molecular target interactions *in vivo*.

The use of the *in vivo* hollow fiber model for pharmacodynamic analysis is consistent with the recent shift observed in drug screening from an empirical to a more rational, molecular targeted approach (49, 50). The current NCI hollow fiber assay does not define any precise mechanisms of action that may have previously been indicated by the recently molecularly characterized *in vitro* human tumor 60-cell line screen from which compounds have progressed. It would seem feasible to follow up any indications of the mechanism of action of a compound using the relatively rapid and cost-effective hollow fiber model.

In conclusion, this study has successfully characterized the hollow fiber assay for investigating pharmacodynamic end points *in vivo*, namely microtubule and cell cycle disruption. Moreover, the disruptive effects of paclitaxel and combretastatin A1 phosphate on cellular microtubules has been demonstrated for the first time *in vivo* using this modified hollow fiber model. Additionally, the *in vivo* hollow fiber assay has demonstrated its use in "proof of principle" studies using the investigational agent combretastatin A1 phosphate.

Although it is appreciated that the hollow fiber assay is used successfully by the NCI as a routine screening model to quantitatively define anticancer activity, it is suggested that the role of this *in vivo* assay could be extended. Ultimately, this study provides evidence to support the routine use of the hollow fiber assay for the demonstration of pharmacodynamic end points *in vivo*.

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