Lack of Multicellular Drug Resistance Observed in Human Ovarian and Prostate Carcinoma Treated with the Proteasome Inhibitor PS-341[1]

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ABSTRACT

Almost all known conventional cytotoxic anticancer drugs are less effective in killing tumor cells grown as multicellular spheroids than in killing tumor cells grown as monolayer cell cultures. This "multicellular resistance" reflects the relative intrinsic drug-resistant phenotype of most solid tumors growing *in vivo* and is due to factors such as limited drug penetration or reduced fractions of proliferating cells. Proteasome inhibitors such as PS-341, a dipeptide boronic acid analogue, represent an interesting new class of potential anticancer drugs, which are entering early-phase clinical trials. PS-341 has been found to have good broad-spectrum cytotoxic activity in the 60-monolayer cell line National Cancer Institute screen. However, because its relative potency has not been tested in spheroid systems, we analyzed the activity of PS-341 in a spheroid/solid tumor context using four different human ovarian carcinoma cell lines and three prostate carcinoma cell lines, respectively. We found, with one exception, that PS-341 showed equal or greater activity in spheroids than in the respective monolayer cell cultures, even in a prostate cancer spheroid model with a very low growth fraction. PS-341 induced apoptotic cell death in carcinoma cells in both culture systems. We also noted a decrease in XIAP protein, a member of the inhibitor of apoptosis (IAP) family of apoptosis inhibitors, and phosphorylation of Bcl-X_L in PS-341-treated ovarian carcinoma cells. Furthermore, DNA fragmentation, a hallmark of apoptosis (in this case, induced by PS-341), was completely inhibited by the caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD). Taken together, the results indicate that unlike most other known anticancer cytotoxic drugs, PS-341 appears to be as effective in killing tumor cells grown in the form of multicell spheroids as in killing tumor cells grown in monolayer cell culture. Hence, this compound has the potential to circumvent multicellular drug resistance and, as such, may show promising activity against solid tumors with low growth fractions *in vivo*, which are frequently intrinsically resistant to conventional cytotoxic anticancer drugs.

INTRODUCTION

Patients whose tumors express resistance to chemotherapeutic drugs tend to do so in one of two ways, either intrinsically or after showing an initial response. The latter is usually referred to as acquired drug resistance. Approximately 60% of cases of drug resistance are of the intrinsic variety and include a broad spectrum of important tumors such as lung, bladder, prostate, and renal carcinomas and melanoma (1). A number of possible mechanisms have been put forward to account for intrinsic drug resistance, including, among others, a lack of sufficient drug penetration into solid tumors, a low growth fraction, or an inherently reduced sensitivity to undergoing drug-induced apoptosis (2, 3).

By growing cells in culture as multicellular tumor spheroids, it is possible to mimic some of the *in vivo* microenvironmental and behavioral characteristics of solid tumors, including hypoxia and intrinsic resistance to chemotherapeutic agents (4–6). For example, significant reductions in the proportion of proliferating tumor cells can result in spheroids with values that are more akin to the cell cycle kinetics of most solid tumors *in vivo* (4–6). Because the majority of conventional cytotoxic anticancer drugs preferentially kill cycling cells, this feature of solid tumors can severely limit the efficacy of such drugs (7, 8). Moreover, some of these drugs may also have a diminished capability to diffuse into the inner layers of multicell spheroids or the tumor parenchyma of a solid tumor *in vivo* (9). Limited penetration can also be a factor in reducing the effectiveness of various immunotherapeutic strategies in the treatment of multicellular spheroids and solid tumors *in vivo* (10–12). Furthermore, hypoxia can also result in spheroids expressing resistance to ionizing radiation (4, 5).

We have been using tumor spheroids as a means of evaluating the properties and relative potential of new anticancer drugs in the treatment of solid tumors. The rationale is that if a drug demonstrates antitumor activity in a spheroid model or screen comparable to or greater than that seen in a conventional monolayer system, it could indicate more promising efficacy for

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the treatment of solid tumors *in vivo*. In other words, the results of the spheroid assay may be of greater predictive value than those of conventional monolayer cell cultures, at least as far as the response of solid tumors is concerned.

With these considerations in mind, we decided to evaluate the relative effects of a new class of anticancer drug, proteasome inhibitors, in monolayer *versus* spheroid cultures. The proteasome represents the cell's major nonlysosomal tool to degrade or process proteins by ATP/ubiquitin-dependent proteolysis (13–15). In higher eukaryotic cells, the proteasome is involved in the degradation of most of the cytosolic proteins, in particular, the short-lived ones, many of which are critical for cell proliferation and cell cycle regulation, as well as apoptosis. Examples include the tumor suppressor protein p53 (16), various cyclins (17), and the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (18, 19). The ordered and temporal degradation of these numerous key proteins is required for cell cycle progression and mitosis; thus, the ubiquitin-proteasome pathway plays a significant role in neoplastic growth, survival, and metastasis. The proteasome is also required for the activation of NF-κB[3] by degradation of its inhibitory protein IκB. NF-κB is required to maintain cell viability through the transcription of inhibitors of apoptosis in response to environmental stress or cytotoxic agents. Stabilization of the IκB protein and blockade of NF-κB activity have been demonstrated to render cells more sensitive to apoptosis (19–22).

Recently, a unique series of proteasome inhibitors that are potent, selective, and reversible in their action have been described by Adams *et al.* (23, 24). These compounds are dipeptide boronic acid analogues (23) that inhibit the chymotryptic activity of the proteasome and, as a result, suppress or block activity of the enzyme (23, 24). One such drug, PS-341, has been found to have an excellent cytotoxic profile and activity when tested on the National Cancer Institute screen or panel of 60 different tumor cell lines, all grown as monolayer cell cultures (24). However, PS-341 has not yet been evaluated in a tumor spheroid context. The results of such studies could be informative with respect to the use and mechanisms of proteasome inhibitors as anticancer agents for solid tumors. To this end, we report here a series of experiments designed to evaluate the cytotoxic (proapoptotic) activity of PS-341 using a panel of seven different human carcinomas (four ovarian and three prostate carcinomas). The results indicate PS-341 has activity against spheroids that is comparable or even superior to that observed in monolayer cell culture systems.

MATERIALS AND METHODS

Tumor Cell Lines and Culture Condition. The HEY human ovarian carcinoma cell line was obtained from Dr. R. Buick (University of Toronto, Toronto, Ontario, Canada). The A2780 human ovarian carcinoma cell line was obtained from Dr. T. Hamilton (Fox Chase Cancer Center, Philadelphia, PA), whereas the OVCA 429 cell line was obtained from Dr. Cindy Boyer (Duke University Medical Center, Durham, NC). The SKOV3 human ovarian carcinoma cell line and the three human prostate cancer cell lines used in these studies, PC3, LNCaP, and DU-145, were obtained from the American Type Culture Collection (Manassas, VA). HEY, A2780, OVCA 429, SKOV3, and LNCaP were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS (Life Technologies, Inc.), and DU-145 and PC3 were grown in DMEM (Life Technologies, Inc.) supplemented with 10% FBS. The cell cultures were maintained in a 37°C humidified incubator with an atmosphere of 5% CO_2. Single cell suspensions were prepared by treatment with trypsin-EDTA (Life Technologies, Inc.) and washing and resuspending in complete medium before setting up monolayer or spheroid cultures.

Multicellular spheroids of the various cell lines were prepared by the liquid overlay technique, as described previously (25). In brief, 24-well or 96-well tissue culture plates (Nunc) were coated with 0.25 ml or 60 μl, respectively, of 1% Seaplaque-agarose (FMC, Bioproducts, Rockland, ME) prepared from a 4% stock solution. For the MTS and [^{3}H]thymidine incorporation assays with the prostate cell lines, an alternate protocol for spheroid culture was used, *i.e.*, 2% poly(2-hydroxyethylmethacrylate) or "polyhema" (Sigma-Aldrich) was used instead to coat U-bottomed 96-well polystyrene multiwell plates (Nunc) as described previously (26). One ml of 10^5 cells or 100 μl of 10^4 cells were added to to each well of the 24-well or 96-well plates, respectively. To allow the cells to come in close contact with one another, the 24-well plates were placed on an orbital shaker and rotated at 250 rpm for 5 min, and the 96-well plates were centrifuged at 1000 rpm for 5 min.

Monolayer culture conditions for the MTS and [^{3}H]thymidine assays were the same as those for spheroid cultures, except that uncoated 96-well tissue culture plates were used, and the plates were not rotated or centrifuged. For all other assays, cells were grown in 100-mm-diameter tissue culture dishes (Nunc) and harvested when approximately 75% confluent.

MTS Staining. Cell viability was assessed essentially as described previously (27). This assay is composed of two solutions: (*a*) a tetrazolium compound (MTS); and (*b*) an electron-coupling reagent (PMS). MTS was purchased from Promega and Fisher Scientific, and PMS was purchased from Sigma-Aldrich. Briefly, cells were cultured in 100 μl of media in 96-well plates for 48 h, and then drugs/compounds were added and incubated for certain time intervals, as indicated. After incubation, 20 μl of the MTS/PMS solution were added to each well and incubated at 37°C for 6 h. MTS is bioreduced by cells into a formazan that is soluble in tissue culture medium. The relative cell viability was obtained by measuring the absorbance of formazan at 490 nm on the Bio-Rad 3550 microplate reader.

[^{3}H]Thymidine Incorporation. The conditions for this assay were essentially the same as those for the MTS assay, except that the cells were pulsed with 50 μl of [^{3}H]thymidine (Amersham Pharmacia) at 40 μCi/ml for 6 h at 37°C. Proliferating cells incorporated the [^{3}H]thymidine into their DNA, which was harvested and collected onto a filtermat using the Titertek Cell Harvester and counted on the Wallac 1205 Beta-

[3] The abbreviations used are: NF, nuclear factor; FBS, fetal bovine serum; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium; PARP, poly(ADP-ribose) polymerase; PMS, phenazine methosulfate; BrdUrd, bromodeoxyuridine; HNFN, 10 mM HEPES (pH 7.4), 150 mM NaCl, 4% FBS, and 0.1% NaN_2; PI, propidium iodide; CDDP, cisplatin; Z-VAD, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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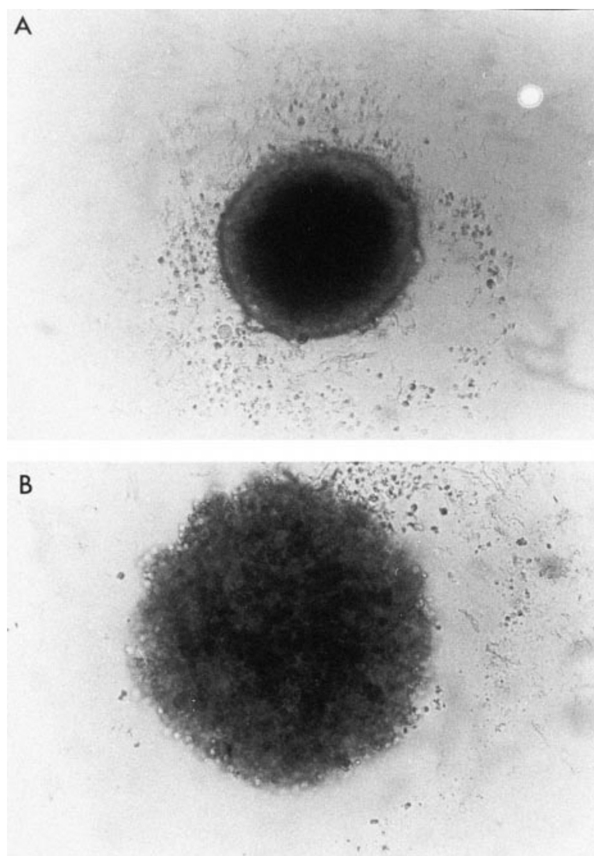

Fig. 1 Light microscopy of OVCA 429 cells incubated for 48 h in RPMI 1640 supplemented with 10% FBS with and without 300 nM PS-341. *A*, control; *B*, PS-341-treated cells. Magnification, ×40.

Plate scintillation counter. Proliferation is expressed as a percentage of the maximum incorporation by the control.

S-phase Analysis by BrdUrd Incorporation. The procedure used in this assay has been described previously (28). Cells grown in monolayer or spheroid culture were pulsed with 10 μM BrdUrd (Sigma-Aldrich) for 3 h. Cells were then rinsed with PBS, trypsinized, rinsed again, and fixed with cold 70% ethanol for at least 1 h on ice. Cells were washed in PBS, resuspended in 1 ml of cold 0.1 N HCl/0.7% Triton X-100, and left on ice for 10 min. The cells were then washed with PBS, resuspended in 0.5 ml of distilled water, and transferred to a 0.5-ml microcentrifuge tube already containing 16 μl of 0.1 N HCl. The tube was then heated in a PCR machine for 8 min at 95°C and placed immediately on ice. Cells were transferred to a 5-ml tube, rinsed twice with HNFN buffer containing 0.5% Tween 20, and rinsed once with HNFN alone. Pellets were then resuspended in a 1:40 dilution of FITC-conjugated anti-BrdUrd antibody (Cedarlane Laboratories) and incubated on ice in the dark for 45 min. Stained cells were rinsed twice with HNFN/Tween 20, rinsed once with HNFN, pelleted, resuspended in 1 ml of PI solution (50 μg/ml PI and 10 μg/ml RNase A in PBS), and incubated for 1 h at 4°C in the dark before analyzing on a Becton Dickinson FAScan flow cytometer.

Flow Cytometry for Detection of Apoptosis. The proteasome inhibitor PS-341 was synthesized by Millennium Pharmaceuticals, Inc. (23, 24). Briefly, cells grown in monolayer culture or in spheroid culture were incubated with varying concentrations of PS-341 for 48 h. Cells were collected, fixed with 1% paraformaldehyde for 15 min, and then fixed with 70% ethanol for 1 h. The Boehringer Mannheim *in situ* cell death detection kit was then used to study apoptosis. The assay is based on terminal deoxynucleotide transferase labeling of DNA strand breaks by the addition of fluorescein dUTP to free 3′-OH DNA ends. The cells were then analyzed by flow cytometry for detection of shifts in fluorescence that indicated increased DNA fragmentation.

Western Blot Analysis. Cells grown in monolayer or spheroid culture were incubated with 5 μM PS-341 and harvested at various time points. Cells were then lysed with lysis buffer supplemented with protease inhibitors (1 mM sodium orthovanadate and 2 mM phenylmethylsulfonyl fluoride). The lysate was then centrifuged at 14,000 rpm for 15 min, and postnuclear supernatant was harvested and sampled for quantitation of protein concentration, using the Bradford dye. The lysate (30 μg) was then mixed with 5× SDS-PAGE sample buffer, boiled for 5 min, and subjected to electrophoresis in 12% SDS gels under reducing conditions. The separated proteins were then electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA). After blocking by TBS-T (5% nonfat dried milk in Tris-buffered saline with 0.125% Tween 20) for 1 h at room temperature, the membranes were blotted with an anti-p21 antibody (Santa Cruz Biotechnology) at a concentration of 0.1 μg/ml, an anti-Bcl-x antibody (Transduction Laboratories) at a concentration of 0.25 μg/ml, an anti-Bax antibody (Santa Cruz Biotechnology) at a concentration of 0.25 μg/ml, and an anti-XIAP antibody (Apoptogen, Inc.) at a 1:2000 dilution. After washing in TBS-T, the membrane was incubated with antirabbit immunoglobulin-horseradish peroxidase (1:1000 dilution) for 1 h. Protein bands were detected by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Cell Death Detection ELISA. The kit for this assay was purchased from Roche Molecular Biochemicals. This is a photometric enzyme-immunoassay for the qualitative and quantitative determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death. Briefly, SKOV3 cells were plated in either monolayer or spheroid cultures at 1×10^5 cells/well in a 24-well dish (Nunc). Forty-eight h later, cells were pretreated overnight with 100 μM Z-VAD (Calbiochem), a caspase inhibitor, and then treated with 5 μM PS-341 in combination with 100 μM Z-VAD for an additional 24 h. Duplicate samples were then collected from the dishes, lysed, and placed into a streptavidin-coated 96-well plate. Subsequently, a mixture of anti-histone-biotin and anti-DNA-peroxidase-conjugated antibodies were added and incubated for 2 h, both of which bind to histone-DNA complexes in the nucleosomes. After removal of unbound antibodies by a washing step, the amount of nucleosomes was quantified by the peroxidase retained in the immunocomplex. Peroxidase was determined photometrically with ABTS (2,2′-azino-di[3-ethylbenzthiazolin-sulfonat] as substrate. The absorbance reading at 405 nm was corrected to a negative control and expressed as an

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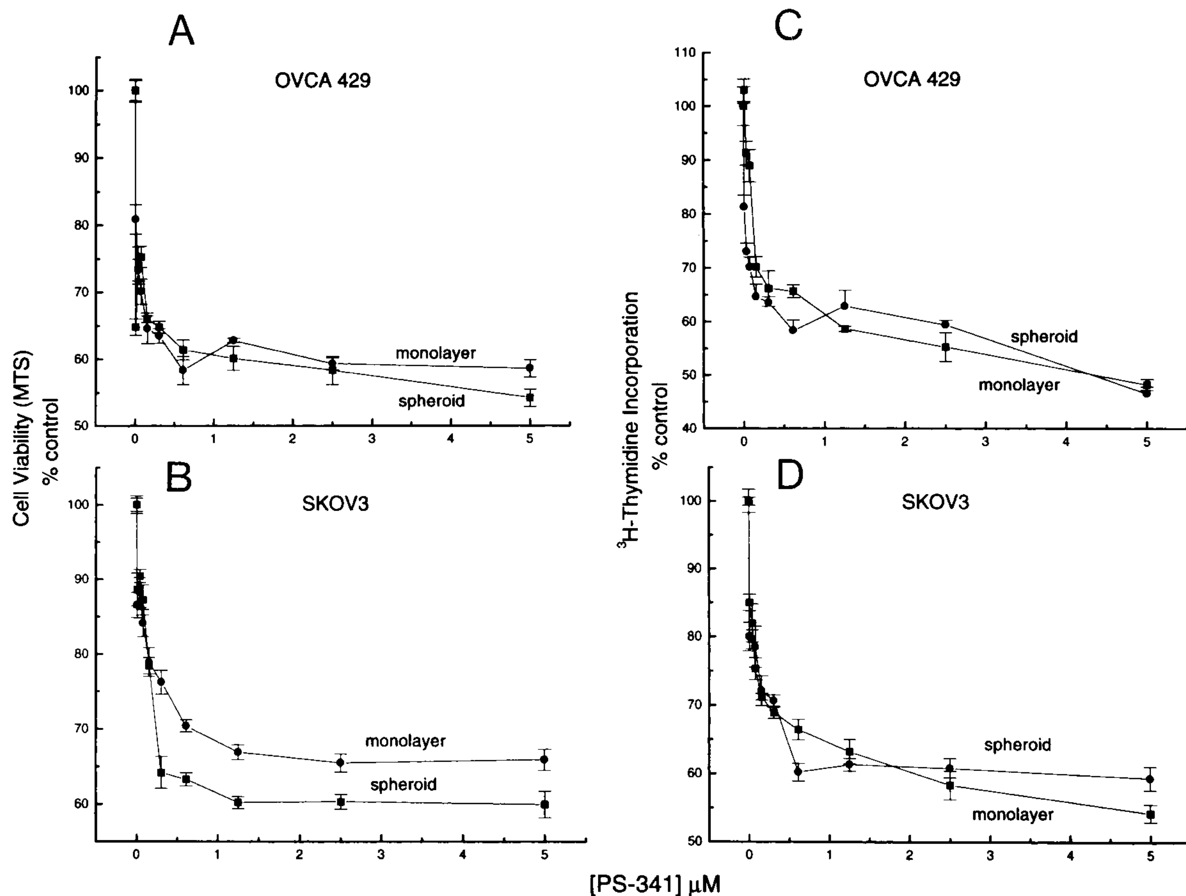

Fig. 2 Comparative effects of PS-341 in inducing cell death (*A* and *B*) and inhibiting cell proliferation (*C* and *D*) in spheroid *versus* monolayer ovarian carcinoma cell cultures as assessed by a MTS cell viability assay and [^{3}H]thymidine incorporation. The cells were plated in spheroid or monolayer cultures in RPMI 1640 supplemented with 10% FBS and incubated with varying concentrations of PS-341 for 48 h.

enrichment factor (or fold increase in DNA fragmentation), according to the manufacturer's instructions.

Drug Sensitivity. The sensitivity of DU-145, PC3, and LNCaP cells to 4-hydroperoxycyclophosphamide (a metabolically active form of cyclosphamide; Nova Pharmaceutical Corp.), Taxol (paclitaxel; Bristol-Myers Squibb), CDDP (Sigma-Aldrich), Adriamycin (doxorubicin hydrochloride; Pharmacia, Inc.), or PS-341 was assessed by the MTS and/or [^{3}H]thymidine assay. The cells were plated at 10^4 cells/well in 100 µl of complete medium and incubated for 48 h, after which 100 µl of the drugs at various concentrations were added, and the plates were incubated for an additional 12 h before pulsing with MTS or [^{3}H]thymidine as described above.

RESULTS

PS-341 Disrupts Intact Multicellular Tumor Spheroids of Human Ovarian Carcinoma Cells. To determine whether human ovarian cancer cells are potential targets for proteasome inhibitors, we first studied the effect of PS-341 on intact spheroids of OVCA 429 ovarian carcinoma cells. Multicellular aggregates were formed from the OVCA 429 cells and cultured in 10% FBS for 48 h. At this time, the intact spheroids were treated with and without PS-341. After a 48-h treatment, the spheroids were observed by light microscopy and photographed. Fig. 1*A* shows OVCA 429 cells grown as a multicellular tumor spheroid without the addition of PS-341, whereas Fig. 1*B* shows the effect of 300 nM PS-341 on a previously intact spheroid. A significant disruption of cellular adhesion in the treated cells was observed. Similar effects were observed with three other human ovarian cancer cell lines, namely SKOV3, HEY, and A2780 (data not shown).

PS-341-induced Toxicity Is Comparable in Both Monolayer and Spheroid Culture. Fig. 2 demonstrates the effect of PS-341 on the viability and tritiated thymidine incorporation of OVCA 429 and SKOV3 ovarian carcinoma cell lines plated in both monolayer and spheroid culture treated over a range of concentrations for 48 h. The viability curves are superimposable when comparing the effects of PS-341 on monolayer *versus* spheroid cultures in the OVCA 429 cell line (Fig. 2*A*). Comparable with the OVCA 429 cell line, the viability curves of the spheroid and monolayer cultures of the SKOV3 cells treated with PS-341 are similar at low nanomolar concentrations of the drug, *i.e.*, at 5 nM PS-341, 88.2% of the spheroid and 86.8% of the monolayer cultured cells

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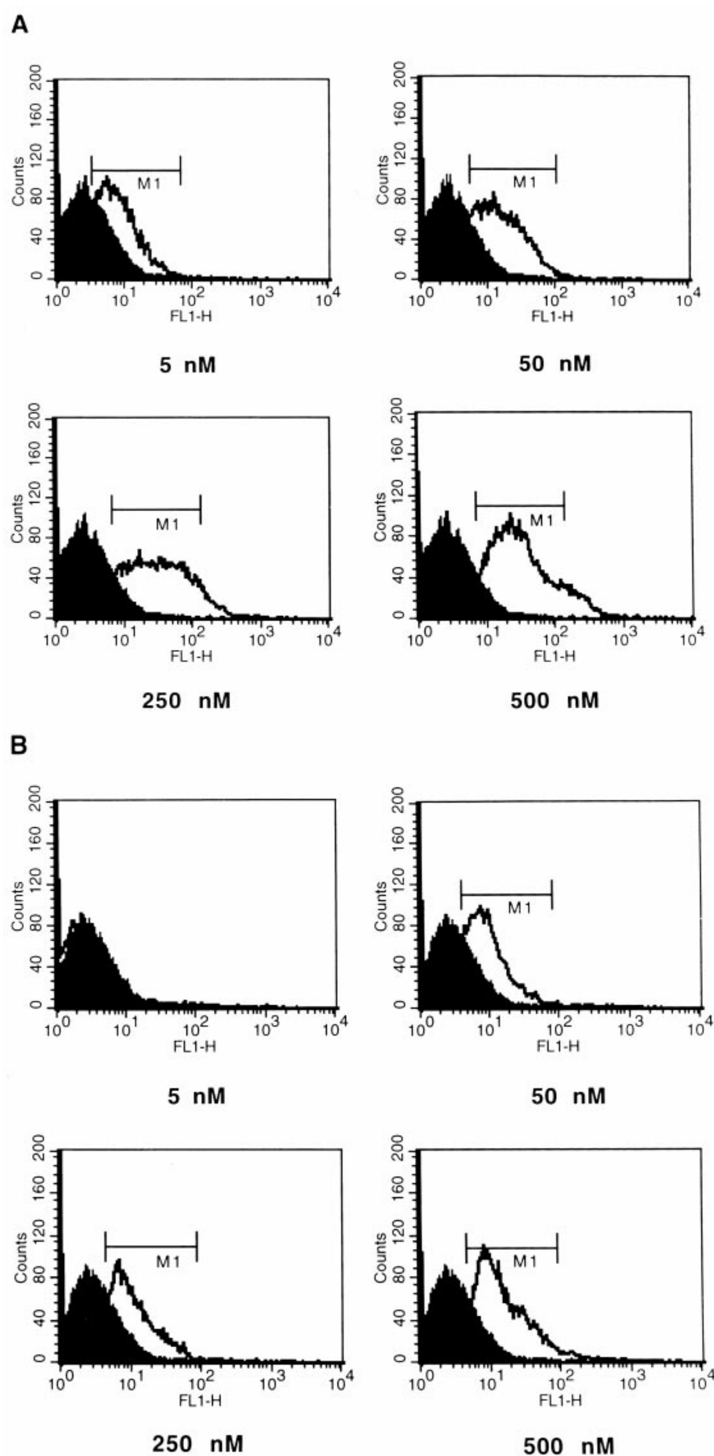

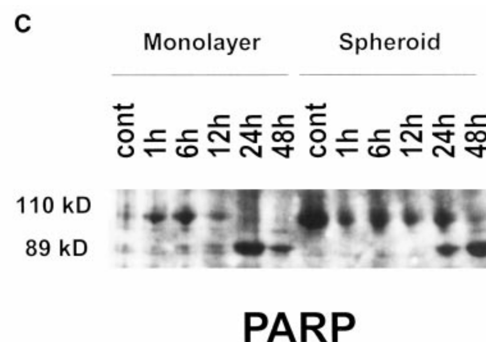

Fig. 3 PS-341 induces apoptosis in both monolayer (*A*) and spheroid (*B*) cultures of ovarian carcinoma cells as represented by the SKOV3 cell line. The cells were exposed to the varying concentrations of PS-341 for 48 h. SKOV3 cells in monolayer or spheroid culture treated with 5 μM PS-341 were harvested at the treatment time indicated, and whole cell lysates were analyzed for the level of PARP protein by Western blot analysis (*C*).

remain viable. However, at concentrations from 300 nM to 5 μM PS-341, the cell viability of the monolayer cultured cells is actually greater than the viability of the cells grown as multicellular tumor spheroids, *i.e.*, at 300 nM PS-341, 75.8% of the cells remain viable in the monolayer cultures compared with 63.8% of the spheroid cultured cells.

These results are in marked contrast to previously published results from experiments conducted in our laboratory using a different drug in which these same cell lines were treated with and without 10 μM Taxol for the same length of time, *i.e.*, 48 h, and a relative resistance to Taxol-induced cell death, as assessed by cell viability, was still observed in the spheroids

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(29). In addition, the Taxol-induced apoptosis detected in monolayer cultures was abrogated in the spheroid culture.

To confirm the MTS results and rule out a possible problem with penetration of the MTS dye into the interior of the spheroid, tritiated thymidine incorporation was measured in both the spheroid and monolayer cultures after PS-341 treatment. As can be observed in Fig. 2, *C* and *D*, there are no significant differences in cell proliferation (as measured by the tritiated thymidine uptake) in the two cell culture systems in either the OVCA 429 cells or the SKOV3 cells.

PS-341 Induces a Comparable Level of Apoptosis in Both Culture Systems. SKOV3 cells were plated in either monolayer or spheroid cultures and exposed 48 h later to varying concentrations of PS-341 for 48 h. In the monolayer culture (Fig. 3*A*), treatment with 5 nM PS-341 induced a shift to the right in the graph, indicative of apoptosis. At this low concentration, no induction of apoptosis was observed in the spheroid culture (Fig. 3*B*). However, at 50, 250, and 500 nM concentrations of the drug, similar levels of apoptosis were observed in both cultures. At 50 nM PS-341, 40.42% of the monolayer cultured cells had undergone apoptosis compared with 43.47% of the spheroid cultured cells. There were comparable increases in the percentage of apoptotic cells with increasing concentrations of PS-341 in both culture systems. Similar results were observed with the HEY and OVCA 429 cell lines (data not shown). To further validate the induction of apoptosis by PS-341 in ovarian carcinoma cells, treatment with the compound was able to cleave PARP into its M_r 89,000 fragment, which is known to serve as an early specific marker of apoptosis (Fig. 3*C*).

Alterations in Various Regulators of Apoptosis after PS-341 Treatment: Increase in p21^{WAF1}, Phosphorylation of Bcl-X$_L$, and Decrease in XIAP in Both Culture Systems. SKOV3 cells plated in monolayer and spheroid cultures were treated with 5 μM PS-341 and harvested at various time points after treatment. There was a conspicuous induction of p21 protein levels (Fig. 4*A*) that were comparable in monolayer and spheroid cultures. After 6 h in both culture systems, the levels began to increase significantly, and by 12 h, there was a strong up-regulation of the protein. The pattern of up-regulation in spheroid culture mirrors that of the monolayer culture. The upper band of the bcl-X$_L$ protein, which represents the hyperphosphorylated form of the protein, becomes more significant after 12 h of treatment with PS-341 in the monolayer culture system and as early as 6 h in the spheroid culture, implying that this antiapoptotic protein becomes inactivated after treatment with the proteasome inhibitor. The levels of the apoptotic protein, bax, did not change, even after a 48 h treatment with PS-341. The level of XIAP, a member of the inhibitor of apoptosis (IAP) family of antiapoptotic proteins, was down-regulated 24 h after PS-341 treatment in both culture systems, and after 48 h, the protein was barely detectable.

PS-341 Appears to Act through the Caspase Pathway. When SKOV3 cells were pretreated with Z-VAD, the caspase inhibitor, and then exposed to the proteasome inhibitor, PS-341, there was a complete abrogation of PS-341-induced DNA fragmentation in both culture systems (Fig. 4*B*). Cells were treated with 100 μM Z-VAD and treated 24 h later with 5 μM PS-341 in combination with Z-VAD to ensure the irreversibility of the caspase inhibitor. The increase in absorbance was indicative of an increase in oligonucleosomal-sized fragments of DNA in the cytoplasm, which is a hallmark of DNA fragmentation. This was measured by a cell death detection ELISA. Z-VAD, on its own, had a protective effect over the cells. There was a 3.4-fold decrease in the level of DNA fragmentation in the monolayer culture and a 2.2-fold decrease in the spheroid culture. PS-341, on its own, induced DNA fragmentation in both culture systems. In the monolayer culture, there was a 2.9-fold increase in DNA fragmentation as compared with the control monolayer cultured cells after PS-341 treatment and a 5.1-fold induction of DNA fragmentation in the spheroid culture. Pretreatment with Z-VAD resulted in complete protection of the cells, indicating that

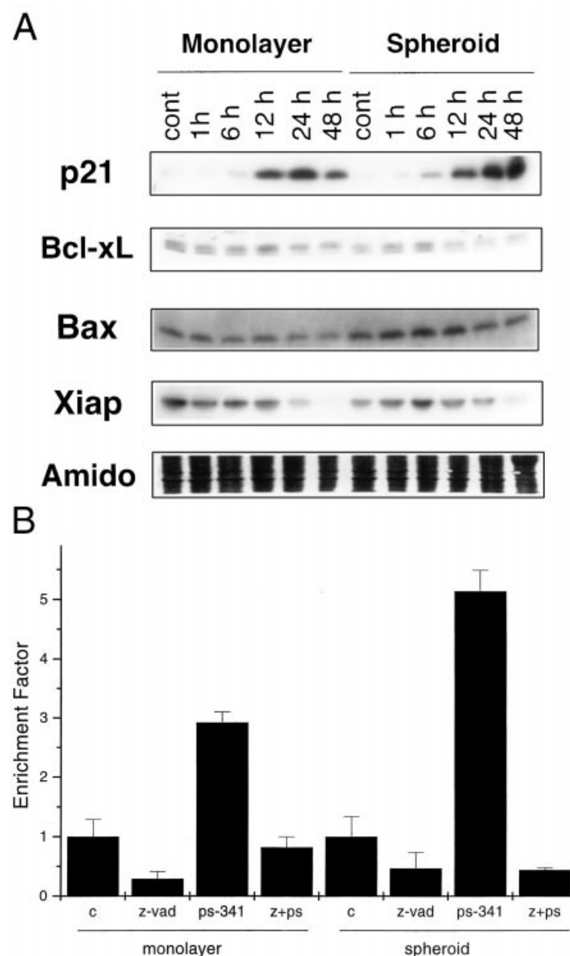

Fig. 4 *A*, the effect of PS-341 over time on various protein levels in the SKOV3 cell line plated in both monolayer and spheroid cultures for 48 h before treatment with the compound. Whole cell extracts were prepared and analyzed by Western blot for expression of the cyclin-dependent kinase inhibitor p21, the antiapoptotic proteins Bcl-X$_L$ and XIAP, and the proapoptotic protein Bax. *Amido* represents the amido black staining of the membrane to determine equal protein loading. *B*, pretreatment of SKOV3 cells with the caspase inhibitor Z-VAD completely abrogates PS-341-induced apoptosis in both spheroid and monolayer cultures. The apoptosis of the cells was quantitated by ELISA. The data represent the average of two independent experiments, each done in duplicate. *c*, control; *z-vad*, treatment with Z-VAD; *ps-341*, treatment with PS-341; *z+ps*, treatment with the Z-VAD and PS-341 combination.

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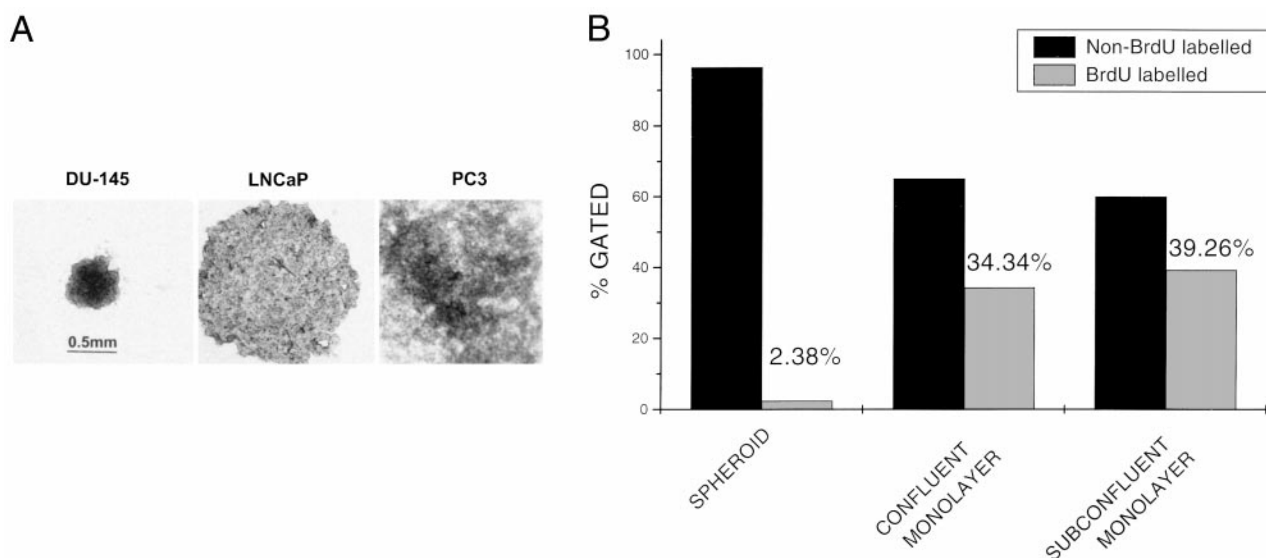

Fig. 5 *A*, light microscopy of prostate cancer cell spheroids (DU-145, LNCaP, and PC3) incubated for 48 h in complete medium. Magnification, ×40. *B*, S-phase analysis of DU-145 grown in spheroid, confluent monolayer, or subconfluent monolayer culture conditions for 48 h and pulsed with BrdUrd for 3 h. Cells were stained with PI just before analysis by flow cytometry.

PS-341 does not induce DNA fragmentation in the apparent absence of caspase activation.

Effects of PS-341 on Human Prostate Carcinoma Cell Lines. We attempted to grow the PC3, LNCaP, and DU-145 human prostate cancer cell lines as multicellular spheroids. Only DU-145 cells were competent to form a highly compact or cohesive spheroid, as shown in Fig. 5*A*. When S-phase analysis was undertaken with DU-145 cells grown in subconfluent or confluent monolayer cell culture, 34.34% and 39.26%, respectively, were found to be BrdUrd positive (Fig. 5*B*). This may help explain the results presented in Fig. 6, which show the relative effects on thymidine incorporation of Taxol, CDDP, 4-hydroperoxycyclophosphamide, or Adriamycin on monolayer cultures (high growth fraction) *versus* spheroids (low growth fractions) of DU-145 cells. Monolayer cell cultures were more sensitive to these conventional cytotoxic drugs, which are known in general to target rapidly dividing cells. These results may help resolve the paradox of Teicher *et al.* (30), who showed that solid tumors derived from the injection of human prostate cancer cell lines into nude mice tend to be resistant to chemotherapy, whereas the same cell lines grown as monolayer cell cultures were found to be relatively drug sensitive (30). The solid tumors *in vivo* would typically have much lower growth fractions than monolayer cell cultures. Spheroids of DU-145 cells, on the other hand, appear to mimic the low growth fractions of metastatic human prostate cancer (8). In light of these results, it is of interest to note the different activity profile that is obtained when PS-341 is used to treat prostate cancer spheroids and monolayer cell cultures. As shown in Fig. 7, PS-341 was actually more effective at the higher doses against DU-145 spheroids than against the corresponding monolayer cell cultures.

DISCUSSION

The proteasome is an intracellular protease recently implicated in several critical cellular functions, including cell cycle progression, antigen-presenting pathways, and apoptosis (31, 32). It is a ubiquitously expressed multisubunit complex of M_r 700,000 that functions to degrade proteins that are targeted by the ubiquitination system (33). Because of its role in tumor cell growth and survival, the proteasome has recently attracted considerable interest as a possible therapeutic target (24, 34, 35); it has also been implicated in the mechanism of action of new drugs such as eponemycin that were not originally designed to block proteasome function (36).

Our results indicate that unlike the majority of anticancer chemotherapeutic drugs, the proteasome inhibitor PS-341 appears to be as effective in killing tumor cells exposed in the form of dense multicellular spheroids as in killing those in subconfluent monolayer cell cultures. Of particular interest was the observation that in DU-145 prostate cancer cells in which the S-phase growth fraction (approximately 2.5%) was more than 1 order of magnitude lower in spheroids than in monolayer cell cultures of the same tumor, the drug was actually *more* effective against spheroids. This suggests that the activity of PS-341 may not be compromised by the relatively low growth fractions characteristic of many types of solid tumor such as prostate cancer, a common feature of many solid tumors that is known to limit the initial effectiveness of many conventional cell cycle-dependent chemotherapeutic agents (4, 5, 7). This feature of PS-341, along with its impressive broad spectrum antitumor activity and its steep dose-response effects, as assessed in the National Cancer Institute 60-cell line screen (24), may bode well for PS-341 and possibly for other proteasome inhibitors as anticancer drugs for the treatment of solid tumors, provided that such drugs have a good therapeutic index, *i.e.*, the toxic effects

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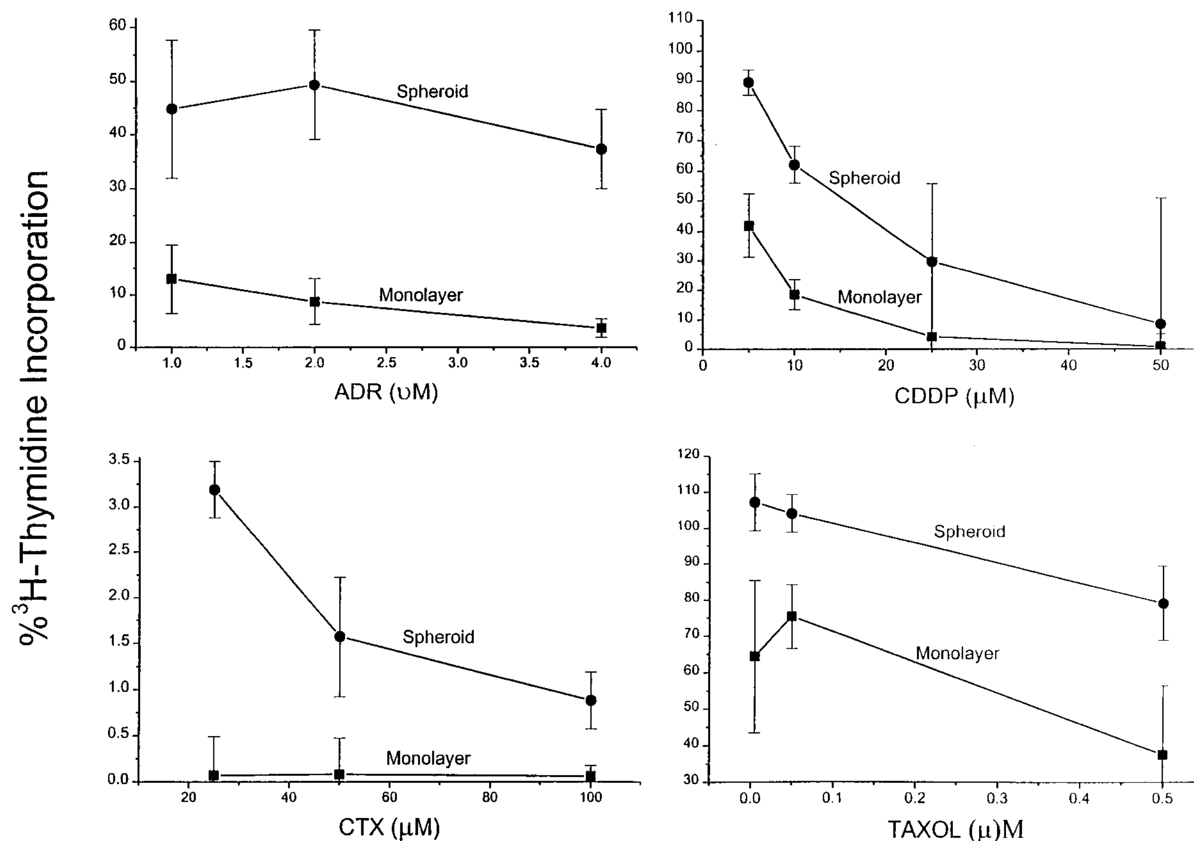

Fig. 6 Relative effects of Taxol, CDDP, cyclophosphamide (*CTX*), and Adriamycin (*ADR*) on thymidine incorporation in monolayer cultures *versus* spheroids of DU-145 cells. The cultures were exposed to the various concentrations of drugs for 12 h and pulsed with tritiated thymidine for 6 h.

to normal tissues of such drugs are not too severe. This is currently being tested in Phase I clinical trials.

Our results suggest that PS-341 treatment induces tumor cell apoptosis and is able to do so equally well in tumor spheroids and monolayer cell cultures. For example, according to the flow cytometry results measuring apoptosis, there are comparable increases in the percentage of apoptotic cells with increasing concentrations of PS-341. To further implicate this apparent induction of apoptosis, treatment with PS-341 gave a similar pattern of PARP cleavage in both culture systems. In addition, phosphorylation of the Bcl-x_L protein is observed in the cells grown as spheroids as well as monolayers, suggesting that this antiapoptotic protein may become similarly inactivated in both culture systems after treatment with PS-341. Furthermore, the level of the antiapoptotic protein XIAP was found to be down-regulated in both culture systems after treatment with PS-341. It is of considerable interest to note that XIAP represents one of the NF-κB-regulated genes (37). Thus, proteasome inhibition resulting in the stabilization of IκB, NF-κB's inhibitory "partner" protein, suggests that the PS-341-induced down-regulation of XIAP may be due to inhibition of NF-κB activation. However, additional experiments to ascertain the direct involvement of NF-κB are clearly required. Finally, we also observed that pretreatment with the caspase inhibitor Z-VAD protects both the spheroid and monolayer cultures, implying that caspase activation may be critical for PS-341-induced apoptosis. These results do not indicate whether the particular regulators of apoptosis we examined are actually directly involved in mediating the cytotoxic (proapoptotic) effects of PS-341 treatment that we have observed.

These results may seem in contrast to several reports in the literature suggesting that proteasome inhibitors are cytotoxic to proliferating cells (38–40). However, in addition to cell cycle proteins, the ubiquitin-proteasome system degrades a number of cell survival proteins, such as Bcl-2 (41) and other members of the Bcl-2 family of apoptotic and antiapoptotic proteins including Bid (42) and Bax (43). Other proteins degraded by this system include STAT3 (44), topoisomerase II (45), and MHC-1 (46) restricted antigens. Moreover, Teicher *et al.* (47) and Adams *et al.* (24) tested the *in vivo* effects of PS-341 in the EMT-6 mouse mammary carcinoma and PC-3 human prostate carcinoma systems, respectively. Teicher *et al.* (47) demonstrated a 90% tumor cell kill by i.p. injection of a single dose of PS-341 in EMT-6 tumors growing s.c. Similarly, Adams *et al.* (24) reported that direct injection of PS-341 into the tumor growing s.c. resulted in a 70% decrease in tumor volume. The proportion of proliferating cells in a spheroid is highly representative of the cell cycle kinetics of most solid tumors *in vivo* (4–6). Therefore, these *in vivo* therapy studies would appear to support out spheroid results.

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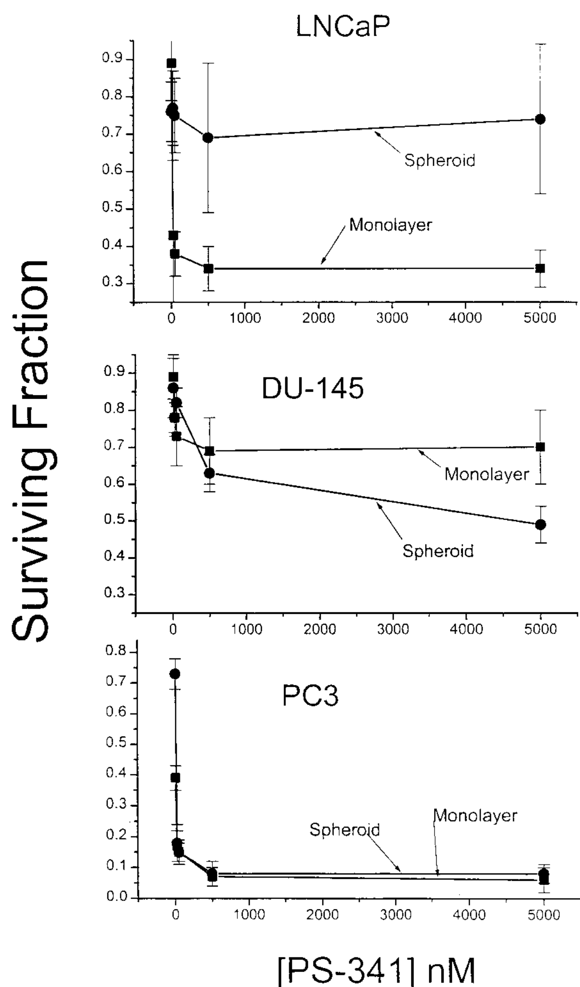

Fig. 7 Effects of PS-341 on the viability of monolayer and spheroid cultures of LNCaP, DU-145, and PC3. The cultures were exposed to varying concentrations of PS-341 for 48 h, as indicated, followed by the addition of MTS for 6 h. The relative cell viability was obtained by measuring the absorbance at 490 nm.

In summary, our results add to a small but growing body of evidence that indicates that the proteasome may be a novel target for anticancer therapy (48). In addition to their potential as anticancer agents on their own (24, 49), proteasome inhibitors can also be used in a combination-type therapy as chemosensitization agents to increase the apoptotic sensitivity of tumor cells to other drugs such as tumor necrosis factor (50). As such, it will be of considerable interest to evaluate the effects of PS-341 on the efficacy of chemotherapy and/or radiation therapy.

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