Cellular Bases of the Antitumor Activity of the Novel Taxane IDN 5109 (BAY59-8862) on Hormone-refractory Prostate Cancer¹

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ABSTRACT

Taxane-based therapies appear to have a significant efficacy in clinical trials on hormone-refractory prostate carcinoma. In the present study, we investigated the cellular response of androgen-independent prostate carcinoma cell lines to the novel taxane IDN 5109 (BAY 59-8862) and evaluated its antitumor activity. In previous preclinical studies, this new paclitaxel (PTX) analogue was characterized by high tolerability and antitumor efficacy, ability to overcome multidrug resistance, and activity by oral administration. Upon treatment, DU145 and PC3 prostate carcinoma cell lines underwent a transient mitotic arrest. This was followed by G1 arrest and rapid occurrence of apoptosis in DU145 cells, whereas in PC3 cells, which are defective for the postmitotic checkpoint, a slow cell death was preceded by DNA endoreduplication. At the biochemical level, such events were associated with tubulin polymerization, activation of the mitosis-promoting factor, and phosphorylation of Bcl-XL/Bcl-2/Raf-1. In addition, IDN 5109 showed an improved pharmacological profile compared with PTX in terms of both efficacy and tolerability, and it revealed a potential therapeutic advantage of IDN 5109 over PTX in human hormone-refractory prostate carcinoma.

INTRODUCTION

Taxanes represent an important class of antitumor drugs that inhibit the functions of cellular microtubules by suppressing their dynamics. They are potent inhibitors of cell growth and cell cycle progression, induce apoptotic cell death, and are endowed with antiangiogenic properties. The two clinical taxane prototypes, PTX¹ and docetaxel, are widely used as components of chemotherapies for ovarian and breast carcinomas and have shown efficacy against a large number of other solid tumors including carcinomas of the lung, head and neck, bladder, and esophagus (1, 2).

Extensive syntheses of new analogues have been performed in the past few years to overcome drug resistance and reduce dose-limiting toxicities, which represent the main limitations to the clinical use of taxanes. From such studies, the semisynthetic IDN 5109 [13-[(N-boc-β-isobutylisoserinyl)-14-hydroxybaccatin-1,14-carbonate] emerged as a molecule of therapeutic interest (3, 4). This PTX analogue was derived from the diterpene 14β-hydroxy-10-deacetylbaccatin III extracted from the needles of Taxus wallichiana (5). In preclinical studies, IDN 5109 showed an improved pharmacological profile compared with PTX in terms of both efficacy and tolerability, and it was characterized by the unique feature, among this class of compounds, of overcoming multidrug resistance (4, 6) and maintaining antitumor activity after oral administration (7, 8). Because of such favorable properties, IDN 5109, now BAY 59-8862, has recently entered Phase I clinical trials.

Because taxane-based therapies have recently shown a significant activity in clinical trials on HRPC (9, 10), the identification of novel, more effective taxanes, as well as the elucidation of the mechanisms of the antitumor activity, might contribute to improve the therapeutic options for such disease.

The purpose of the present study was to investigate the cellular response of human androgen-independent prostate carcinoma cell lines to IDN 5109 and to evaluate its antitumor efficacy in hormone refractory prostate xenografts. We examined cell cycle perturbations, modulations of apoptotic and angiogenic factors, cell growth inhibition, and cell death. Despite a com-

¹ The abbreviations used are: PTX, paclitaxel; HRPC, hormone-refractory prostate cancer; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; MMP-2, mitotic protein monoclonal-2; TW, tumor weight; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PI, propidium iodide; BrdU/rd, bromodeoxyuridine; P-gp, P-glycoprotein.

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parable behavior of IDN 5109 and PTX at the cellular level, the results support the therapeutic interest of the novel taxane for HRPC.

MATERIALS AND METHODS

Drugs and Cell Culture. IDN 5109 and PTX were provided by Indena S.p.A. (Milan, Italy). The synthesis and chemical structure of the new analogue IDN 5109 were reported previously (5, 6). The two taxanes were dissolved in ethanol at 1 mg/ml and further diluted in culture medium.

The human androgen-independent prostate carcinoma cell lines PC3 and DU145 were maintained in RPMI 1640 (Bio-Whittaker, Verviers, Belgium) supplemented with 10% FCS (Life Technologies, Inc., Gaithersburg, MD). For experiments, cells were treated 24 h after seeding with solvent or with different concentrations of taxanes for 24 h and then incubated in drug-free medium for the indicated times before processing for specific tests. In experiments performed to evaluate the expression of VEGF and bFGF, cells were left in the presence of the drug for 72 h. Unless stated otherwise, pools of floating and adherent cells were used in the assays.

Western Analysis. After treatment with taxanes for the indicated times, cells were collected, and cell lysates were prepared as described previously (11). Equal amounts of proteins were separated by SDS-PAGE and then transferred onto nitrocellulose filters. After staining with Ponceau-S (Sigma, St. Louis, MO) to control protein loading and transfer, filters were processed for Western blot as reported previously (12). The following primary antibodies were used: monoclonal anti-cyclin B1 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Bcl-2 (Dako, Glostrup, Denmark); anti-Bcl-X1 (PharMingen, San Diego, CA) and MPM-2 (Mitotic Protein Monoclonal-2, Upstate Biotechnology, Lake Placid, NY); rabbit polyclonal anti-p34cdc2, anti-Raf-1, anti-Cdc25C, anti-VEGF, anti-bFGF (Santa Cruz Biotechnology); anti-cdc2 [Tyr15-phosphospecific; New England BioLabs, Beverly, MA]; and anti-tubulin (BioMakor, Rehovot, Israel). Immunocomplexes were visualized by the Amersham (Little Chalfont, United Kingdom) enhanced chemiluminescence procedure or Pierce (Rockford, IL) Super Signal system.

Tubulin Polymerization Assay. Cells were treated with IDN 5109 or PTX (60 nM) for 24 h. Then, fractions containing soluble cytosolic and polymerized tubulin were obtained as described previously (12). Tubulin distribution in the cell fractions was detected by Western blotting using a rabbit antitubulin antibody.

p34cdc2 Kinase Assay. Cells were treated with the indicated concentrations of taxanes for 24 h. p34cdc2 kinase activity was detected in cell lysates, in the presence of [γ-32P]ATP, as the ability to phosphorylate a specific peptide substrate, according to the Amersham Biostack cdc2 kinase enzyme assay kit. Experiments were performed in duplicate.

Cell Cycle Analysis. Taxane-induced cell cycle perturbations were analyzed, following PI staining of DNA, as reported previously (13). At least 10,000 cells were collected and evaluated for DNA content. Cell cycle distributions were calculated by LYSYS II software (Becton Dickinson).

Biparametric flow cytometric analysis of BrdUrd incorporation was performed as described previously (12). Green fluorescence from secondary FITC-conjugated antibody and red fluorescence from PI were detected as a measure of BrdUrd incorporation and DNA content, respectively.

Evaluation of Cell Death and Multinucleation. At the indicated times after drug treatment (60 nm, 24 h), positivity to the TUNEL assay was measured as an index of cell death according to the working procedures of the in situ cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany). Multinucleated cells were evidenced by PI staining performed as described above.

The number of TUNEL-positive and multinucleated cells, as detected by fluorescence microscopy, was assessed on at least 100 cells in two different smears and referred to the whole cell population.

Antiproliferative Assays. Cell sensitivity to taxanes was evaluated by cell and colony growth inhibition assays. In the cell growth inhibition assay, PC3 and DU145 cells were seeded into 6-well plates in duplicate and exposed to IDN 5109 or PTX for 24 h. Adherent cells were counted 72 h after the beginning of treatment by a cell counter (Coulter Electronics, Luton, United Kingdom).

In the clonogenic assay, 400 cells were seeded in 60-mm plates (Corning Costar, Corning, NY) in triplicate and treated for 24 h. Plates were then incubated in drug-free medium until colonies in control samples consisted of around 25 cells. Colonies were stained with 1% crystal violet in methanol for 30 min and counted by an inverted microscope.

IC50s were defined as drug concentrations causing a 50% decrease in cell or colony number over that of untreated controls.

In Vivo Studies and Tumor Vessel Density Evaluation. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori (Milan, Italy) according to the United Kingdom Coordinating Committee on Cancer Research Guidelines (14).

For experiments, DU145 cells from in vitro cell culture were inoculated s.c. in both flanks of athymic mice (107 cells/ flank). Each control or drug-treated group included four mice bearing bilateral s.c. tumors. Tumor growth was followed by biweekly measurement of tumor diameters with a Vernier caliper. TW was calculated according to the formula: TW (mg) = tumor volume (mm3) = d2 × D/2, where d and D are the shortest and the longest diameters, respectively. Drug treatment started when the mean TW was 200 mg. Taxanes dissolved in absolute ethanol (5%) and Cremophor ELP (5%) dissolved in water (90%) according to previously reported procedures (4) were administered i.v. every 4th day, three times, at a dose of 54 and 90 mg/kg (IDN 5109).

For vessel density evaluation, an immunohistochemical technique was used. Zinc-fixed, paraffin-embedded DU145 prostate cancer tissue sections were mounted on poly-l-lysine-coated slides. Blood vessels were stained using a rat antimuscle CD31 monoclonal antibody (kindly supplied by Dr A. Vecchi; Milan, Italy; Ref. 15). Antibody immunodetection was performed with a standard avidin-biotin technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Microvessel density, evaluated in at least five microscopic fields selected as
being highly vascularized, was expressed as the average number of microvessels/field (×400 magnification).

RESULTS

IDN 5109-induced Tubulin Polymerization in DU145 and PC3 Cells. To characterize the response of the two androgen-independent prostate carcinoma cell lines DU145 and PC3 to IDN 5109, we first investigated the effects of the new taxane on the tubulin microtubule system. As evidenced in Fig. 1, after 24 h of treatment, IDN 5109 induced tubulin polymerization to an extent comparable with that induced by the same concentration (60 nM) of the parent drug PTX in both DU145 and PC3 cells. Such a finding is consistent with a similar mechanism of interaction of the two drugs with the primary cellular target.

Activation of Mitosis Factors. The ability to induce mitotic arrest is a hallmark of microtubule inhibitors (16). We examined such an effect at the biochemical level in DU145 cells exposed to the new inhibitor IDN 5109. Cells were exposed to IDN 5109 or PTX for 24 h. As illustrated in Fig. 2A, phosphorylation of Cdc25C phosphatase, dephosphorylation of p34\textsuperscript{cdc2} kinase at Tyr\textsuperscript{15}, and up-regulation of cyclin B1 could be detected in treated DU145 cells by Western blot analysis, thereby indicating an accumulation of cells with an activated cyclin B1/p34\textsuperscript{cdc2} complex (mitosis-promoting factor; Ref. 17). IDN 5109-induced mitotic arrest was further confirmed by the increased reactivity of cell lysates to the MPM-2 antibody, which recognizes several mitosis-specific epitopes (Ref. 18; Fig. 2A), and by the increased p34\textsuperscript{cdc2} kinase activity in treated cells as compared with control cells (Fig. 2B).

We showed previously that PC3 cells had already exited mitosis after 24 h of exposure to PTX due to a less stringent PTX-activated mitotic checkpoint compared with DU145 cells (13). Accordingly, identical results were obtained with IDN 5109 (data not shown).

Raf-1 and Bcl-2/Bcl-X\textsubscript{L} Phosphorylation. A hyperphosphorylated state of Raf-1 and Bcl-2 family members is a biochemical hallmark of the cellular response to microtubule inhibitors (19, 20). The two prostate cancer cell lines DU145 and PC3 display a differential expression of the antiapoptotic proteins Bcl-2 and Bcl-X\textsubscript{L} (13, 21). In DU145 cells, which lack Bcl-2 expression, drug treatment induced a parallel mobility shift of Bcl-X\textsubscript{L} and Raf-1 in SDS-PAGE, thereby indicating a drug-induced phosphorylation of the two proteins (Ref. 22; Fig. 3). Such an effect was comparable with that induced by PTX. Similarly, both drugs induced phosphorylation of Raf-1 and Bcl-2 in PC3 cells, whereas the phosphorylation of Bcl-X\textsubscript{L} protein was not modulated by taxane treatment.

Cell Cycle Perturbations. Cell cycle perturbations induced by IDN 5109 were examined in DU145 and PC3 cells that display a different setting of cell cycle checkpoints activated by microtubule damage, with the main difference being the lack of a functional postmitotic checkpoint in the PC3 cell line (13). The analysis of DNA profiles by flow cytometry indicated that, similar to exposure to PTX, exposure to IDN 5109 for 24 h induced an accumulation of tetraploid cells and the appearance of a sub-G\textsubscript{1} population in the DU145 cell line, which is endowed with a functional postmitotic checkpoint. In contrast, in the postmitotic checkpoint-defective PC3 cell line, hyperploid cells (DNA > 4 N) were evident at 72 h with both drugs, and the appearance of fragmented DNA was delayed to 96 h (Fig. 4).

To confirm that the PC3 hyperploid cell population was the result of S-phase reentry, a dual parameter analysis was performed to monitor actively synthesizing DNA. As shown in Fig. 5, BrdUrd incorporation was detected in the treated cells with a
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DNA content of >4 N, as well as in the expected DNA duplication from 2 N to 4 N, thereby indicating cell cycle progression and DNA endoreduplication.

Cell Death, Multinucleation and Inhibition of Cell Proliferation. Because the analysis of cell cycle perturbations induced by IDN 5109 in DU145 and PC3 cells suggested a different cellular response, cell death was investigated in treated cell lines over time. In parallel, drug-induced multinucleation, an effect that has been related to taxane cytotoxicity (23), was determined by PI staining of nuclei. As shown in Fig. 6A, IDN 5109 (60 nM) induced cell death, detected as TUNEL-positive drug-induced DNA fragmentation, in about 30% of the DU145 cell population by 48 h and, similar to PTX, such an effect was maintained at 72 h. In contrast, in PC3 cells, the increase in TUNEL positivity induced by both drugs was minimal and did not exceed 15% up to 72 h. A different level of multinucleation distinguished IDN 5109-treated cells from PTX-treated cells (Fig. 6B). In fact, PTX induced a maximum of about 10% and 25% of multinucleated cells in PC3 and DU145 cells, respectively, after 48 h of treatment, whereas IDN 5109-induced multinucleation reached about 40% at 24 h in both cell lines. These results indicated that cell death preceded by multinucleation occurred at a higher rate after treatment with IDN 5109 as compared with PTX.

The antiproliferative activity of IDN 5109 was compared with that of PTX on DU145 and PC3 cells by a cell growth inhibition assay. In addition, because the different mode of cell death of the two cell lines could influence the evaluation of chemosensitivity, we also assessed the clonogenic survival. As shown in Table 1, the efficacy of the new taxane was similar to that of PTX in both assays. Moreover, the two drugs exhibited comparable IC50 values in the two cell lines.

Together, these results showed a comparable antiproliferative activity by IDN 5109 and PTX on the two prostate cancer cell lines and suggested that such activity was not affected by the modality of undergoing cell death by a rapid or slow process, preceded or not preceded by multinucleation.

Modulation of VEGF and bFGF Expression. The expression of VEGF and bFGF was examined by Western blotting in the DU145 cell line, which express high levels of the two proteins. The effect of taxane treatment is illustrated in Fig. 7. Both growth factors were down-regulated after exposure of the cells to IDN 5109 or PTX (60 nM, 72 h). The similar efficacy of the two drugs indicated that IDN 5109 shares with the parent drug the ability to reduce the expression of these two potent angiogenic factors.

Effects on DU145 Tumor Growth and Angiogenesis. The superior antitumor efficacy of IDN 5109 versus PTX at their respective maximal tolerated dose against the DU145 tumor xenograft was reported previously (4). In this study, the two taxanes affected tumor growth differently, even at the same dose (54 mg/kg). Indeed, the effect was superior and more persistent after IDN 5109 treatment than after PTX treatment (Fig. 8).

In consideration of the results reported above, the in vivo antitumor activity of IDN 5109 was not fully explained by its cellular pharmacology. Thus, we investigated the effect of IDN 5109 on tumor neovascularization, an effect that has been reported to contribute to the in vivo efficacy of taxanes (24, 25). Tumor vessel density was evaluated 7 days after the last treatment with IDN 5109 (54 and 90 mg/kg) or PTX (54 mg/kg). Fig. 9 indicates that treatments with both drugs had significant antiangiogenic activity compared with controls after the first week of treatment. Moreover, in IDN 5109-treated mice, the reduction of tumor vessels was dose dependent and significantly more marked than that in PTX-treated mice (P < 0.05 and P < 0.01 for treatments at 54 and 90 mg/kg, respectively).

DISCUSSION

In the present study, we have shown that IDN 5109, a novel taxane that recently entered clinical trials, is an effective growth inhibitor of human androgen-independent prostate cancer cell lines growing either in vitro cell cultures or as tumor xenografts. The new taxane analogue displays an efficacy comparable with that of PTX in in vitro cell systems and a superior antitumor activity on nude mice bearing an established xenograft of human DU145 prostate cancer cells.

IDN 5109 was initially selected for preclinical investigation because of its ability to overcome cellular resistance mediated by the cell membrane efflux pump P-gp (4, 6), a major factor responsible for chemoresistance to taxanes (26). According to such a property, our previous study (12) showed a correlation between the relative antiproliferative activity of IDN 5109 and PTX and their biochemical effects in two P-gp-expressing ovarian carcinoma cell lines. However, it was unclear whether IDN 5109 was inherently more potent than the parent drug, or whether the different drug potencies were due to P-gp expression. In the two prostate carcinoma cell lines used in the current study (DU145 and PC3, both of which lack P-gp expression; Ref. 27), a comparable level of tubulin polymerization was induced by the two taxanes (Fig. 1). As expected, characterization of the cellular effects of the new analogue showed the hallmarks of antimicrotubule agents, including the induction of a mitotic arrest associated with activation of the mitosis-promoting factor and phosphorylation of Bcl-XL/Bcl-2/
Raf-1. In keeping with a comparable efficacy in such effects, the antiproliferative and cytotoxic activities of IDN 5109 appeared to be similar to those of the parent drug in both cell lines. Our results thus support that IDN 5109 affects the same biochemical pathways involved in the cellular response to PTX, resulting in a comparable cytotoxicity in the absence of P-gp expression.

Furthermore, our data indicate that, as described previously for PTX (13), the in vitro antiproliferative activity of IDN 5109 on the two prostate cancer cell lines does not seem to be affected by the modality of cell death, which was different in the two cell lines. In particular, whereas DU145 cells appeared to activate a classical apoptotic pathway in response to the taxane, PC3 cells

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**Fig. 4** Cell cycle analyses of DU145 and PC3 cells treated with IDN 5109 or PTX at 60 nM for 24 h. Cell cycle perturbations were analyzed by flow cytometry of PI-stained cells at 72 and 96 h after the beginning of treatment. The profiles are from one experiment representative of three independent experiments.

**Fig. 5** Dual parameter analysis of BrdUrd incorporation and PI staining in PC3 cells. Cells were exposed to BrdUrd for 1 h at the time points and then processed as indicated in “Materials and Methods” and counterstained with PI. Fluorescence was determined by FACSscan analysis. FITC fluorescence (BrdUrd incorporation) is plotted on the ordinate, and PI staining (DNA content) is plotted on the abscissa. The profiles are from one experiment representative of two experiments.
underwent a slow cell death characterized by low TUNEL positivity and preceded by DNA endoreduplication. As a unique differential effect compared with PTX in in vitro experiments, IDN 5109 induced a higher percentage of multinucleation in both cell lines. The bases of such effect are not clear at present. Although a correlation was suggested between multinucleation and an attenuated caspase activation (23), our data do not appear to support such a relationship. Indeed, high levels of multinucleation were observed in both cell lines despite a different propensity to undergo drug-induced apoptosis.

Experimental evidence has been provided that inhibition of angiogenesis could play a role in the antitumor activity of taxanes (24, 25). Although the mechanisms underlying such an effect are not fully understood, it has been suggested that the down-regulation of VEGF, a potent angiogenic growth factor produced by tumor cells (28), could contribute to the antiangiogenic properties of PTX (29). In this study, we have found that both PTX and IDN 5109 effectively reduced the expression of VEGF in DU145 prostate cancer cells in vitro. In addition, we have demonstrated that both drugs reduced the expression of
bFGF, another major angiogenic growth factor (28). These findings might be of relevance to the therapy of HRPC because dysregulation of growth factors, including VEGF and bFGF, has been suggested to be involved in the development of prostate tumors, and emerging evidence indicates that prostate cancer growth and metastasis are angiogenesis dependent (30–32). Moreover, a clinically relevant role has been ascribed to VEGF as an angiogenesis promoter in prostate cancer. In fact, its expression in tumors has been positively correlated with tumor stage, grade, microvessel density, and clinical outcome (33), whereas urinary VEGF levels have been proposed as an independent prognostic factor, with high levels predicting poor prognosis (34). It is noteworthy that both VEGF and bFGF appear to play a dual role in the prostate cancer microenvironment, being involved in autocrine loops and paracrine actions and thus regulating tumor cell proliferation as well as endothelial cell functions and neovascular development (31, 35). In this respect, an inhibitory effect of taxanes on the expression of the two angiogenic factors, as observed in in vitro-treated DU145 cells (Fig. 7), might affect prostate cancer growth in vivo by acting both directly on tumor cells and indirectly on the tumor neovascularization.

The cellular and biochemical response to IDN 5109 did not fully explain the superior antitumor activity of IDN 5109 as compared with PTX against the DU145 tumor xenograft. In vivo studies indicated a superior inhibitory activity of IDN 5109 versus PTX on tumor vessel density (Fig. 9). The effect was dose dependent and was more marked at 90 mg/kg IDN 5109 than at 54 mg/kg IDN 5109. However, the similar efficacy of the two taxanes in vitro, including down-regulation of endogenous VEGF and bFGF, suggests that additional, as yet unrecognized mechanisms could account for the increased efficacy of IDN 5109 in vivo. Such mechanisms might involve direct effects on endothelial cells affecting their motility and invasiveness, as described previously for PTX (24). An alternative explanation could be a favorable pharmacokinetic behavior, as indicated by the long half-life reported for IDN 5109 in mice plasma (7, 8).

Taken together, our results support a therapeutic advantage of the new taxane IDN 5109 over the parent drug PTX against HRPC, associated with more efficient inhibition of tumor neo-vascularization.

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