PD-0332991, a Potent and Selective Inhibitor of Cyclin-dependent Kinase 4/6, Demonstrates Inhibition of Proliferation in Renal Cell Carcinoma at Nanomolar Concentrations and Molecular Markers Predict for Sensitivity

JOSHUA E. LOGAN[1], NIKAYEH MOSTOFIZADEH[2], AMRITA J. DESAI[2], ERIKA VON EUW[2], DYLAN CONKLIN[2], VEERAUO KONKANKIT[2], HABIB HAMIDI[2], MARK ECKARDT[2], LEE ANDERSON[2], HSIAO-WANG CHEN[2], CHARLES GINTHER[2], EILEEN TASCHEREAU[2], PETER H. BUI[3], JAMES G. CHRISTENSEN[4], ARIE S. BELLDEGRUN[1], DENNIS J. SLAMON[2] and FAIROOZ F. KABBINAVAR[1,2]

[1]*Institute of Urologic Oncology, Department of Urology, David Geffen School of Medicine, UCLA, Los Angeles, CA, U.S.A.;*
[2]*Division of Hematology/Oncology, Department of Medicine, David Geffen School of Medicine, UCLA, Los Angeles, CA, U.S.A.;*
[3]*Molecular Cytogenetics Laboratory, Department of Pathology, David Geffen School of Medicine, UCLA, Los Angeles, CA, U.S.A.;*
[4]*Pfizer Global Research and Development, Pfizer Oncology, San Diego, CA, U.S.A.*

Abstract. *Background: PD-0332991 is an inhibitor of cyclin-dependent kinases (CDK) 4 and 6, and was evaluated to determine its anti-proliferative effects in 25 renal cell carcinoma (RCC) cell lines. Materials and Methods: Half-maximal inhibitory concentrations (IC_{50}) of PD-0332991 were determined with cell line proliferation assays, as were its effects on the cell cycle, apoptosis, and retinoblastoma (RB) phosphorylation. Molecular markers for response prediction, including p16, p15, cyclin D1 (CCND1), cyclin E1 (CCNE1), E2F transcription factor 1 (E2F1), RB, CDK4 and CDK6, were studied using array comparative genomic hybridization (CGH) and gene expression. Results: IC_{50} values for PD-0332991 ranged from 25.0 nM to 700 nM, and the agent demonstrated G_0/G_1 cell-cycle arrest, induction of late apoptosis, and blockade of RB phosphorylation. Through genotype and expression data p16, p15 and E2F1 were identified as having significant association between loss and sensitivity to PD-0332991: p16 (p=0.021), p15 (p=0.047), and E2F1 (p=0.041). Conclusion: PD-0332991 has antiproliferative activity in RCC cell lines, and molecular markers predict for sensitivity to this agent.*

Correspondence to: Joshua E. Logan, MD, Institute of Urologic Oncology, Department of Urology, David Geffen School of Medicine, University of California, Los Angeles, 924 Westwood Blvd., Suite 1050, Los Angeles, CA 90095, U.S.A. Tel: +1 3102061434, Fax: +1 3107943513, e-mail: Joshuaelogan@gmail.com

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Cell-cycle dysregulation is prevalent in multiple malignancies, including renal cell carcinoma (RCC) (1, 2). Progression from G_1 to S phase is an important checkpoint in regulating cell proliferation. This transition is mediated through the retinoblastoma protein (RB), which is regulated through sequential phosphorylations by cyclin-dependent kinases (CDK) (1, 3). When RB is sequentially phosphorylated it disassociates from E2F transcription factor-1 (*E2F1*) and *E2F1* then promotes transcription of genes necessary for cell-cycle progression (4). In order for RB to be phosphorylated, there must be assembly of the active cyclin D–*CDK4/6* subunit, which is negatively regulated by the Inhibitors of CDK4 (INK4) tumor suppressor family members (5).

The *p16* tumor suppressor, an INK4 family member, has received significant attention as it has shown to be aberrant in many types of human tumors (6, 7). This aberrancy can be secondary to deletions, mutations in the coding region, or promoter methylation (8-10). Evaluation of *p16* in RCC has shown consistent findings. Sanz-Casla *et al*. demonstrated loss of heterozygosity (LOH) in nine out of 39 (23%) primary RCC (11). In another report, homozygous deletions were observed in eight out of 34 (24%) RCC specimens (12). Hypermethylation of the *p16* promoter region has been reported to be as high as 20% (11). Regarding mutations, a rate of 36/372 (10%) of RCC cases has been reported (www.sanger.ac.uk/genetics/CGP/cosmic). The role of other INK4 family members, such as *p15*, are not as well-described.

It is important to note that *RB* is expressed in 95% of RCCs (13). The implication is that cell-cycle progression

Table I. *Cell line names, histological type, cell line origin, as well as comparative genomic hybridization (CGH) copy number status of cyclin-dependent kinase inhibitor 2A (CDKN2A) and cyclin-dependent kinase inhibitor 2B (CDKN2B).*

Cell line	Cell type	Obtained from	*CDKN2A* CGH status	*CDKN2B* CGH status
RCC-JW	Clear cell RCC	Cell Line Services™	Homozygous deletion	Homozygous deletion
RCC-MF	Clear cell RCC	Cell Line Services™	Homozygous deletion	Homozygous deletion
RCC-MH	Clear cell RCC	Cell Line Services™	Homozygous deletion	Homozygous deletion
RCC-WK	Clear cell RCC	Cell Line Services™	No change	No change
RCC-KP	Clear cell RCC	Cell Line Services™	No change	No change
RCC-GW	Clear cell RCC	Cell Line Services™	Homozygous deletion	Homozygous deletion
RCC-JF	Clear cell RCC	Cell Line Services™	Homozygous deletion	Homozygous deletion
RCC-HB	Clear cell RCC	Cell Line Services™	No change	No change
RCC-AB	Clear cell RCC	Cell Line Services™	Homozygous deletion	Homozygous deletion
RCC-ER	Clear cell RCC	Cell Line Services™	No change	No change
RCC-FG1	Clear cell RCC	Cell Line Services™	No change	No change
Caki-1	Clear cell RCC	ATCC™	Homozygous deletion	Homozygous deletion
Caki-2	Clear cell RCC	ATCC™	Homozygous deletion	Homozygous deletion
R11	Clear cell RCC	Gift from Dr. Arie Belldegrun	No change	No change
R444	Clear cell RCC	Gift from Dr. Arie Belldegrun	Homozygous deletion	No change
UMRC6	Clear cell RCC	Gift from Dr. Bart Grossman	Homozygous deletion	Homozygous deletion
UOK 121	Clear cell RCC	Gift from NCI	No change	No change
RCC-EW	Renal adenocarcinoma	Cell Line Services™	Homozygous deletion	Homozygous deletion
769-P	Renal adenocarcinoma	ATCC™	No change	No change
786-O	Renal adenocarcinoma	ATCC™	Homozygous deletion	Homozygous deletion
A-704	Renal adenocarcinoma	ATCC™	Homozygous deletion	No change
ACHN	Renal adenocarcinoma	ATCC™	Homozygous deletion	No change
RCC-PR	Renal carcinoma	Cell Line Services™	Homozygous deletion	Homozygous deletion
Hs 891.T	Renal carcinoma	ATCC™	No change	No change
SW 156	Hypernephroma	ATCC™	Homozygous deletion	Homozygous deletion
CCD 1103	Non-malignant kidney epithelia	ATCC™	No change	No change
CCD 1105	Non-malignant kidney epithelia	ATCC™	No change	No change
HEK-293	Non-malignant kidney epithelia	ATCC™	No change	Unknown

remains dependent upon *RB* and upstream regulatory molecules are viable candidates for targeting the cell-cycle signaling pathway for therapy.

PD-0332991 is an orally active, potent, and selective inhibitor of CDK4 and CDK6, which blocks RB phosphorylation at low nanomolar concentrations (14). This agent, also known as palbociclib, was recently shown to substantially improve progression-free survival by greater than 18 months in patients with estrogen receptor-positive breast cancer and as a result obtained an FDA breakthrough designation.

We evaluated PD-0332991 in multiple RCC cell lines to determine its effects on cell proliferation, the phosphorylation of *RB* in selected sensitive and resistant cell lines, as well as on the cell cycle and apoptosis. In addition, the differential response to this drug was evaluated for association with copy number alterations and variance in transcript expression to identify potential molecular markers of response.

Materials and Methods

Cell lines, cell culture, and reagents. PD-0332991 was examined for its effects on growth inhibition in a composite cell line panel representative of RCC, comprising 28 cell lines, 25 of which are malignant RCC lines and three are non-malignant, transformed cell lines derived from kidney epithelium. The individuality of each cell line was confirmed by mitochondrial DNA sequencing. Each cell line with its histological type, origin and comparative genomic hybridization (CGH) copy number status regarding cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase inhibitor 2B (*CDKN2B*) are described in Table I.

Proliferation assays. Cells were plated in duplicate in 24-well tissue culture plates at a density of 15×10^3 and grown with and without decreasing concentrations of PD-0332991, starting at 1 μmol/l followed by 12 serial 2:1 dilutions. After six days of exposure to the drug, the cells were harvested by trypsinisation and counted using a particle counter (Z1; Beckman Coulter Inc., Brea, CA, USA). Each of these experiments was completed at least twice.

Growth inhibition was calculated as a function of the number of generations inhibited in the presence of PD-0332991 *versus* the number of generations over the same time course in the absence of PD-0332991.

Annexin V and propidium iodide flow cytometry. The effect of PD-0332991 on apoptosis and cell cycle were assessed by annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (MBL International, Woburn, MA, USA) and nuclear isolation medium–4,6-diamidino-2-phenylindole dihydrochloride (NIM-DAPI) staining (NPE Systems, Pembroke Pines, FL, USA)

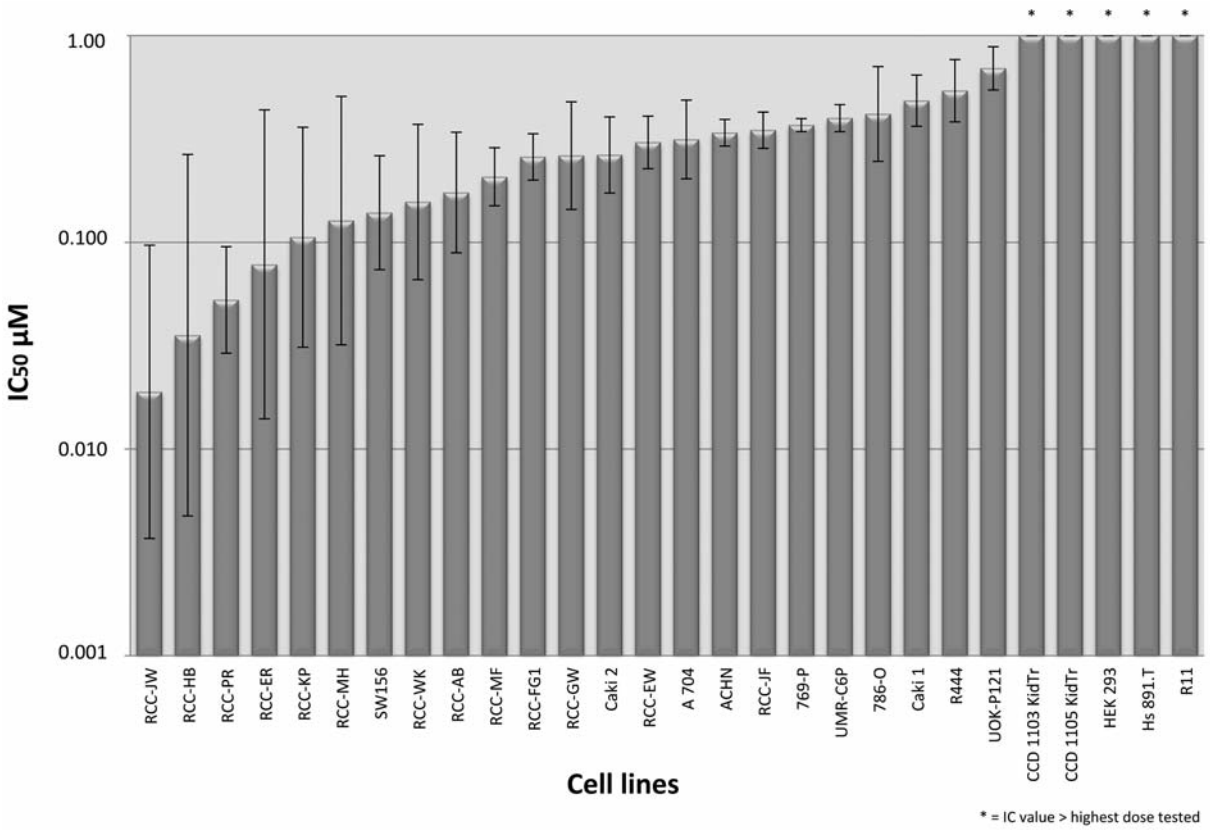

Figure 1. *Inhibitory effects of PD-0332991 on cell proliferation. Cell lines were grown with PD-0332991 (doses ranging 0.0005-1.00 µM). Cell lines are ordered from left to right with low to high RCC-JW, RCC-HB, RCC-PR, RCC-ER, RCC-KP, RCC-MH, SW 156, RCC-WK, RCC-AB, RCC-MF, RCC-FG1, RCC-GW, Caki 2, RCC-EW, A 704, ACHN, RCC-JF, 769-P, UMR-C6P, 786-O, Caki 1, R444, UOK-P121, CCD 1103, CCD 1105, HEK 293, Hs 891.T, R11 (IC_{50}) values. Bars represent the 95% confidence interval.*

respectively and analyzed by flow cytometry. Cells were grown to log phase and then were either treated with 0.1 µM PD-0332991 for 48 hours or received no treatment and served as controls. Flow cytometry was performed using a Cell Quanta (Beckman Coulter Inc.) flow machine, and the results were analyzed using Flow Jo software (Tree Star Inc., Asland, OR, USA).

Western blot and immunoprecipitation. Cells in log-phase growth were treated with 100 nM PD-0332991 and then harvested at 30 minutes, 1 hour, 8 hours, 24 hours, and 48 hours by washing in PBS and lysed at 4˚C in 400 µl of mild lysis buffer. Insoluble material was cleared by centrifugation at 14,000 rpm (16,873 ×*g*) for 10 min at 4˚C. Protein was quantitated using bicinchoninic acid (BCA) (Pierce Protein Research Products, Rockford, IL, USA), resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Life Technologies, Grand Island, NY, USA). Phospho-RB expression was detected by polyclonal anti-pRB (Ser780), and RB expression detected by monoclonal anti-RB (Cell Signaling Technology, Beverly, MA, USA). Detection was performed using ECL Plus chemifluorescent reagent (GE Healthcare, Pasadena, CA, USA).

Gene expression profiling of RCC cell lines. Microarrays for 26 cell lines were completed with the Agilent Human 44K array chip (Agilent Technologies, Santa Clara, CA, USA), as described in an earlier report (15). The reference mix was comprised of equal amounts of RNA from 26 cell lines. Slides were read using the Agilent Scanner and expression values were calculated using Agilent Feature Extraction software version 10.7. Expression data analysis was completed on the Rosetta Resolver system for version 7.2 (Rosetta Biosoftware, Seattle, WA, USA). Intensity ratios between individual cell line samples and the mixed reference were calculated according to the Agilent error model. Sequences were considered expressed differentially if the calculated *p*-value was ≤0.01.

DNA isolation and array CGH. Extraction of genomic DNA from cell lines was completed using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA). Labeling and hybridization of Agilent 105K oligonucleotide CGH arrays were performed according to the manufacturer's protocol for Human Genome CGH 105A Oligo Microarray Kit, Version 5.0 (Agilent Technologies). Files were extracted using Agilent Feature Extraction software v9.5 with the default CGH protocol. Extracted arrays with a DRL Spread <0.3 were included in the analysis. CGH Analytics software v.4.0 (Agilent Technologies) was used for copy number analysis, employing the ADM2 algorithm (Threshold 5), with fuzzy zero and centralization corrections to minimize background noise. All map positions were based on the March 2006 NCBI36/hg18 genome assembly

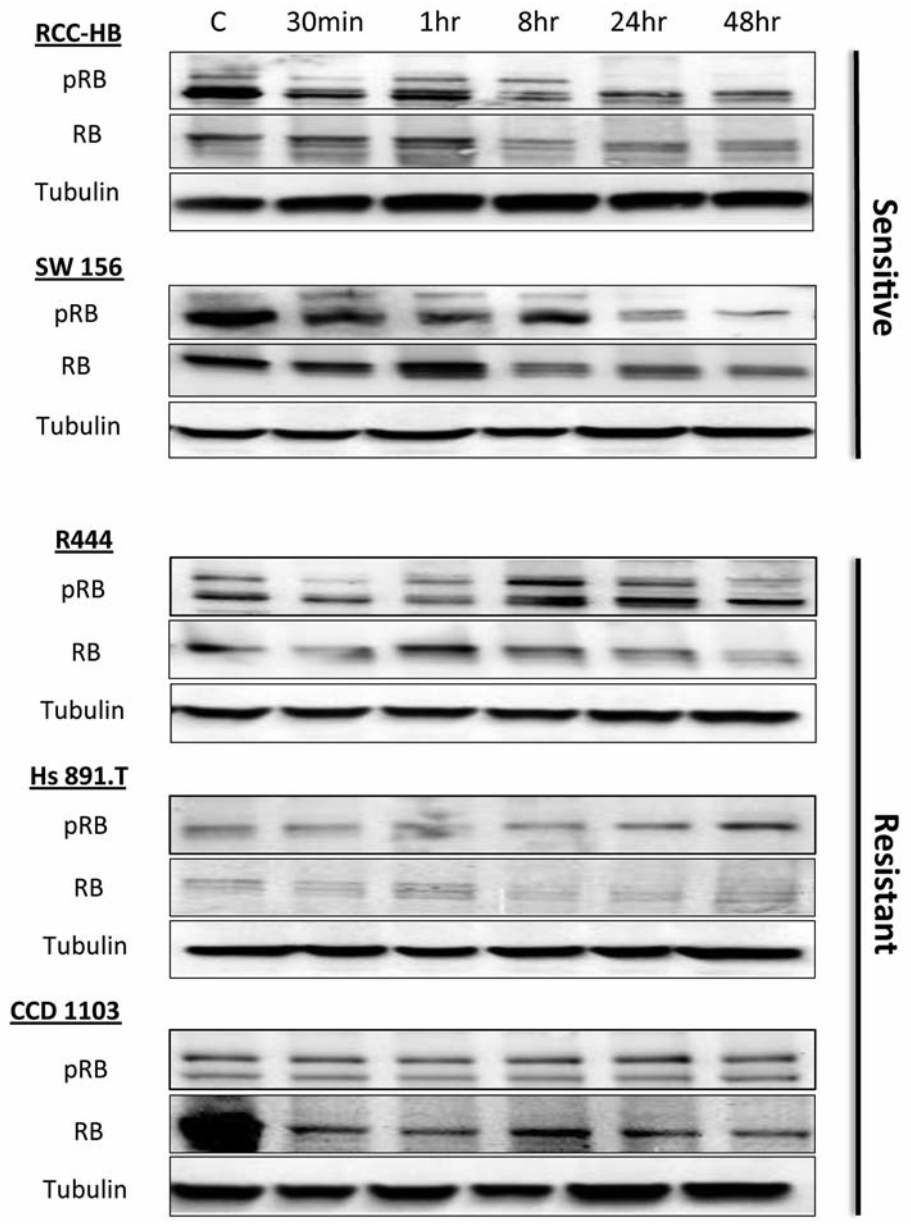

Figure 2. *Effects of 100 nM PD-0332991 at five time points on phosphorylated-RB (pRB) and total-RB (RB). PD-0332991 does not have significant effects on the expression of total-RB in sensitive or resistant cell lines. PD-0332991 blocks phosphorylation of RB in sensitive cell lines (IC_{50}<150 nM), but not in resistant malignant or non-malignant cell lines (IC_{50}>500 nM).*

(http://genome.ucsc.edu/ENCODE/search.html). A minimum of three consecutive probes were required to define a region as amplified or deleted. All data were inspected visually using the interactive view. Log2 ratios larger than 1 were considered amplified (2-fold increase), larger than 2 highly amplified, smaller than -1 were considered hemizygous and smaller than -2 homozygous deletions.

Results

The 50% inhibitory concentration (IC_{50}) of PD-0332991 for each cell line was evaluated using a range of PD-0332991 concentrations: 0.5-1000 nM. PD-0332991 inhibited the proliferation of many of the RCC lines in a wide, concentration-dependent fashion with IC_{50} values of 25 nM in the RCC-JW cell line ranging up to 700 nM for the UOK 121 cell line. In five of the cell lines the IC_{50} value was not reached within the range of concentrations tested, suggesting that it's value exceeds 1.00 μM: CCD 1103, CCD 1105, HEK-293, Hs 891.T and R11 (Figure 1).

The sensitivity threshold for this study was defined as ≤400 nM based upon pharmacokinetic data published from

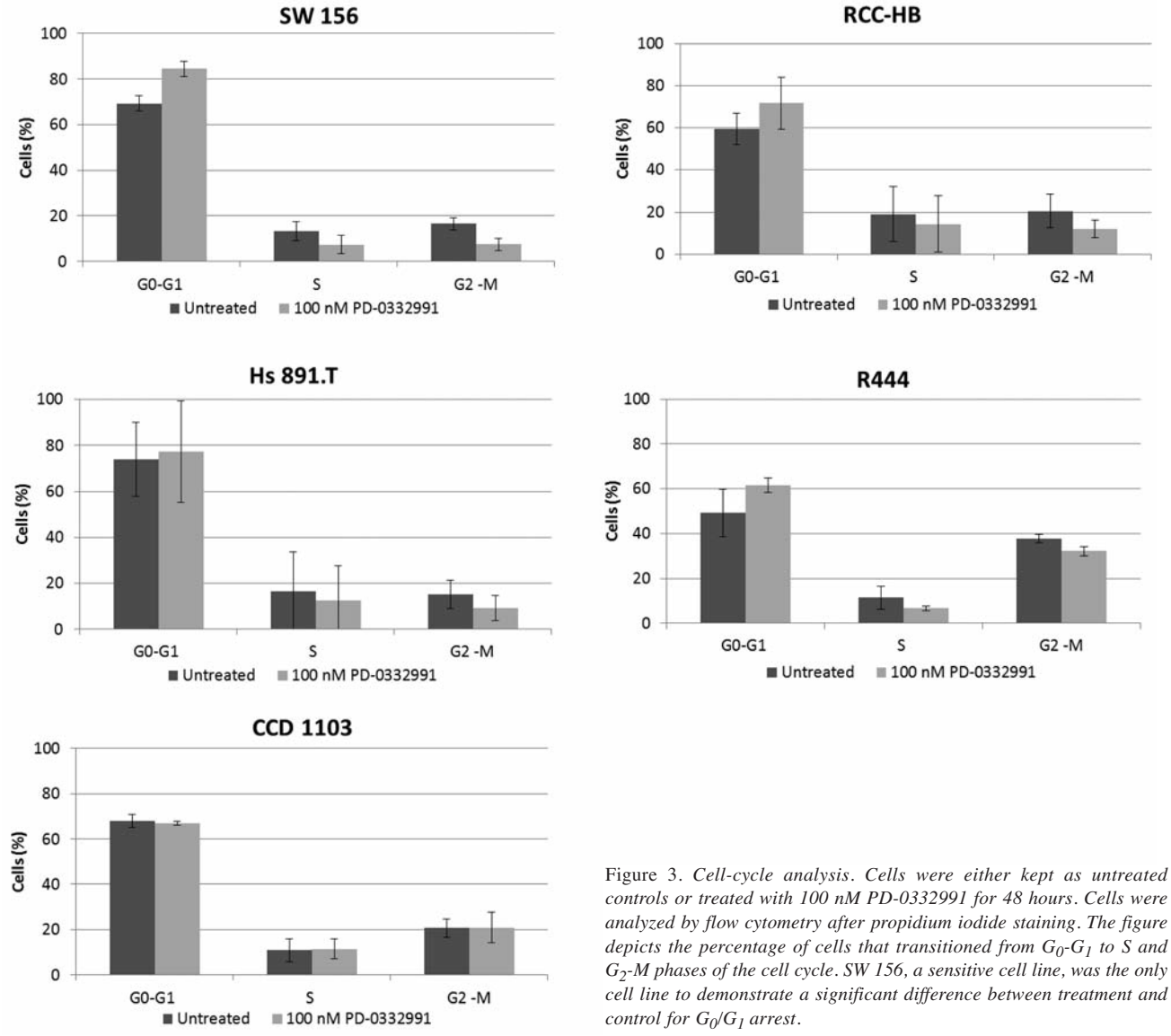

Figure 3. *Cell-cycle analysis. Cells were either kept as untreated controls or treated with 100 nM PD-0332991 for 48 hours. Cells were analyzed by flow cytometry after propidium iodide staining. The figure depicts the percentage of cells that transitioned from G_0-G_1 to S and G_2-M phases of the cell cycle. SW 156, a sensitive cell line, was the only cell line to demonstrate a significant difference between treatment and control for G_0/G_1 arrest.*

a phase I trial in which the peak plasma concentration of PD-0332991 associated with the maximally tolerated dose was slightly greater than 400 nM (16). At this threshold, 19 cell lines were considered to be sensitive and nine were considered to be resistant to PD-0332991.

The effects of PD-0332991 on the phosphorylation of RB were evaluated with western blot at five time points on two sensitive cell lines (RCC-HB and SW 156), two resistant cell lines (R444 and Hs 891.T), and one non-malignant transformed cell line (CCD 1103).

A differential time-dependent profile was seen for the cell lines with regard to phospho-*RB*, where sensitive cell lines were shown to have a decrease in phospho-*RB* with time in the presence of 100 nM PD-0332991, in contrast to the three resistant cell lines, which showed sustained presence of phospho-*RB* despite the presence of 100 nM PD-0332991. Total-*RB* remained relatively constant at each time point in each of the five cell lines after treatment with 100 nM PD-0332991 (Figure 2).

The effects of PD-0332991 on the cell-cycle and apoptosis were evaluated in cell lines at a treatment concentration of 100 nM. After 48 hours of exposure, SW 156 demonstrated G_0/G_1 arrest compared to the control, while RCC-HB and the three resistant cell lines tested, R444, Hs 891.T and CCD 1103, did not show differences in cell cycle arrest compared with the controls (Figure 3).

Differences in apoptosis between treatment and controls were only detected in the late apoptotic population in the sensitive cell line SW 156; no other differences were observed in the other four cell lines evaluated (Figure 4).

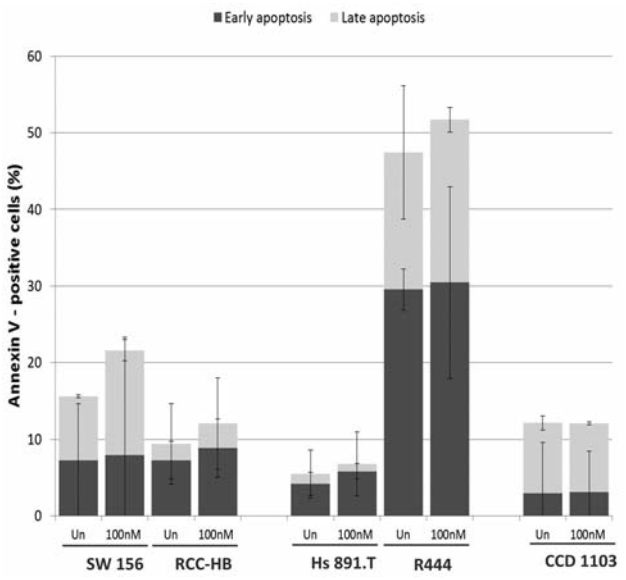

Figure 4. *Apoptosis analysis. Cells were either kept as untreated controls (Un) or treated with 100 nM PD-0332991 for 48 hours. Identification of apoptotic subpopulations were completed by labeling phosphatidylserine residues of the cell surface with annexin V-FITC and staining cells with propidium iodide. This staining allows differentiation of apoptotic subpopulations. SW 156, a sensitive cell line, was the only cell line to demonstrate a significant difference between treatment and control in apoptosis, specifically the sub-population of late apoptosis.*

Copy number alterations were assessed by CGH microarrays, and calls regarding changes were made as described in Materials and Methods. In addition, microarrays were completed for gene expression profiling of the cell lines as described.

Regarding *CDKN2A* (*p16*) and *CDKN2B* (*p15*), homozygous deletions of both genes were very common. Genotype strongly correlated with mRNA expression. Genotype and expression data were combined to make a qualitative consensus call on *p16* and *p15* loss for all 28 lines. One line (769) did not have *CDKN2A* deletion by CGH, but had an mRNA expression level comparable to that of the *CDKN2A*-deleted lines and this was thus classified as a loss.

Both *p15* and *p16* loss were tested for association with response to PD-0332991 using Fisher's exact test. Cell lines with *p16* and *p15* losses were significantly more likely to be classified as responders to PD-0332991 (p=0.027, relative risk (RR)=2.06 and p=0.047, RR=1.71, respectively). Due to their close chromosomal proximity, *CDKN2A* and *CDKN2B* are commonly co-deleted. We observed four lines that had homozygous deletions of *CDKN2A* but not *CDKN2B*, and three of these lines were classified as being sensitive to PD-0332991. There were no lines with only *CDKN2B* deleted. These observations imply that *CDKN2A* is the more likely driver of differential response to treatment, and *CDKN2B* represents a passenger copy number alteration.

There were no observed amplifications or deletions in any of the other cell-cycle genes investigated, hence only mRNA expression levels could be analyzed for an association with response. For each gene, cell lines were classified as either 'high'- or 'low'-expressing based on whether the log mRNA expression(fold change) was above or below zero (*i.e.* expression was above or below the average in the mixed reference). The only gene whose expression was significantly associated with response to PD-0332991was *E2F1* (p=0.041, RR=0.48) (Figure 5).

Discussion

Targeting the cell cycle for cancer therapy has gained significant attention. Inhibition of CDKs has been the target of choice in this biochemical pathway. However, to date in RCC, investigation has been limited to non-specific inhibition of CDKs, which has not ultimately lead to clinical development (17). The focus on targeting the cell cycle has since shifted to inhibition of specific CDKs with the subsequent development of the potent and selective CDK4/6 inhibitor, PD-0332991. With promising results emerging for other malignancies, such as breast cancer, where recent results from a phase II study demonstrated a greater than 18-month improvement in progression-free survival with this novel agent, pre-clinical investigation in RCC, given its mechanistic applicability as outlined in the introduction, is a logical progression and the study presented here is the first on RCC.

The proliferation assays showed a differential response to PD-0332991 with inhibition of growth occurring in the nanomolar range. Inspection of phospho-RB by western blot confirmed the expected decline with time in the two representative sensitive cell lines. Of these two sensitive cell lines evaluated, only SW 156 showed G_0/G_1 arrest at the 100 nM concentration, while RCC-HB showed the expected time-dependent decline in phospho-RB, but no difference in the G_0/G_1 arrest between the treatment and control. This apparent discrepancy between the IC_{50} of RCC-HB as being sensitive at 100 nM, the decrease in phospho-RB with time, and the lack of G_0/G_1 arrest may be reflective of the true IC_{50} being higher in the confidence interval than the mean which is represented in Figure 1, and while PD-0332991 has some effect on the molecular biology at 100 nM partially blocking phosphorylation of RB, it appears this is insufficient to induce cell cycle arrest at this concentration in this cell line.

For the three cell lines defined as being resistant to PD-0332991, concordance was shown between their IC_{50} values, the presence of phospho-Rb on the western blot and lack of G_0/G_1 arrest.

In the genotype/expression response association analysis, deletion and decreased expression of *p16* was identified as a

Cell line	Response	*p16*	*p15*	*CCND1*	*CCNE1*	*E2F1*	*RB1*	*CDK4*	*CDK6*
JW	Sensitive	Yes	Yes	High	High	Low	Low	High	Low
HB	Sensitive	No	No	Low	Low	Low	Low	Low	High
PR	Sensitive	Yes	Yes	High	High	Low	Low	Low	Low
ER	Sensitive	No	No	High	Low	Low	Low	Low	Low
KP	Sensitive	No	No	High	Low	Low	High	Low	High
SW156	Sensitive	Yes	Yes	High	High	High	Low	High	High
WK	Sensitive	Yes	Yes	Low	High	Low	Low	High	High
AB	Sensitive	Yes	Yes	High	High	Low	High	Low	Low
MF	Sensitive	Yes	Yes	Low	Low	Low	Low	High	Low
MH	Sensitive	Yes	Yes	Low	Low	Low	Low	High	Low
FG1	Sensitive	No	No	Low	Low	Low	High	Low	High
GW	Sensitive	Yes	Yes	High	High	Low	Low	High	Low
Caki-2	Sensitive	Yes	Yes	Low	High	High	Low	Low	Low
A704	Sensitive	Yes	No	Low	Low	Low	High	High	Low
ACHN	Sensitive	Yes	No	Low	High	Low	High	Low	Low
JF	Sensitive	Yes	Yes	High	Low	Low	High	High	High
769	Sensitive	Yes	No	Low	Low	Low	High	High	High
UMR	Sensitive	Yes	Yes	Low	Low	High	Low	High	Low
EW	Sensitive	Yes	Yes	High	Low	Low	Low	Low	High
786-O	Resistant	Yes	Yes	High	Low	High	High	High	High
Caki-1	Resistant	Yes	Yes	Low	Low	Low	Low	Low	Low
R444	Resistant	Yes	No	High	High	High	Low	High	High
UOK	Resistant	No	No	High	Low	High	High	High	High
R11	Resistant	No	No	High	High	High	Low	High	Low
Hs 891.T	Resistant	No	No	High	Low	Low	Low	Low	Low
HEK 293	Resistant	No	No	Low	High	High	Low	High	Low
CCD 1103	Resistant	No	No	Low	High	Low	Low	High	High
CCD 1105	Resistant	No	No	High	Low	Low	Low	Low	Low
*p-V*alue		0.021	0.047	0.348	0.910	0.033	0.448	0.491	0.910
RR		2.08	1.71	0.78	0.97	0.47	1.23	0.83	0.97

Figure 5. *Genotype and expression data of cyclin-dependent kinase inhibitor-2A (CDKN2A) and cyclin-dependent kinase inhibitor-2B (CDKN2B) were combined and a consensus was made regarding the status of p16 and p15 (Yes=loss of p16/p15; No=no loss of p16/p15); significant association between loss and sensitivity to PD-0332991 was identified for p16 (p=0.021; RR:2.08) and p15 (p=0.047; RR 1.71). For six other genes involved in the cell cycle, cyclin D1 (CCND1), cyclin E1 (CCNE1), E2F transcription factor 1 (E2F1), retinoblastoma (RB), cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6), cell lines were classified as having 'high' or 'low' expression as there were no amplifications of deletions. E2F1 was the only gene identiifed with expression significantly associated with response to PD-0332991 (p=0.033; RR=0.47).*

significant predictor of sensitivity to PD-0332991. This suggests that PD-0332991 can re-establish the negative regulation of the cyclin D–CDK4/6 complex despite loss of *p16* regulation. The mRNA expression response analysis yielded low expression of *E2F1*, the transcription factor that is bound by RB and released as RB is sequentially phosphorylated, as a significant predictor of response. This suggests that when a low concentration *E2F1* is present and the phosphorylation of RB is blocked, such as by PD-0332991, then the concentration of non-phosphorylated-RB is higher and therefore more available to bind *E2F1*, thereby blocking cell-cycle progression. The expression of other integral genes in this pathway such as *CDK4, CDK6, CCND1, CCNE1,* and *RB* were not identified as predictors of response in this model. This collectively demonstrates that PD-0332991 at nanomolar concentrations selectively inhibits growth of a subset of RCC cell lines which are characterized by loss of one or more key regulators of the cell cycle: *p16* and *E2F1*.

Similar findings have been reported in the pre-clinical work with PD-0332991 in ovarian and breast cancer, as well as other malignancies. Konecny *et al*. showed that *p16* deletions were more common in ovarian cancer cell lines sensitive to PD-0332991, and a clinical trial for this tumor type is now enrolling (18). Similarly, Finn *et al*. showed that in breast cancer cell lines, sensitivity to PD-0332991 was associated with lower levels of *p16* expression (15). This collective work highlights the association between dysregulation in the cell-cycle signaling pathway, with consistent loss of *p16*, and sensitivity to PD-0332991.

The significance of the loss of *p16* or loss of its coding region has been studied at the clinical level. Ikuerowo *et al*. demonstrated in Cox regression analysis that p16 expression is an independent covariate in disease-specific survival in a cohort of 397 patients with RCC (19). Sanjmyatav *et al*. identified five recurrent chromosomal aberrations that were significantly associated with RCC metastasis; one of the most prominent of them with the highest odds ratio for metastatic risk was loss of 9p21.3p24.1, which covers the coding region for *p16* (20). In addition, other authors have also shown that deletion of the 9p region is associated with poor cancer-related outcomes in patients with RCC (21, 22).

Lastly, an appealing aspect of this agent is the possibility of a molecular marker in the cell-cycle pathway from which responders could potentially be identified. Overall, these findings along with reported pre-clinical work in other malignancies and positive results in clinical trials, support further investigation of PD-0332991 in RCC.

Conflicts of Interest

There is no conflict of interests.

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