PCK3145 Inhibits Proliferation and Induces Apoptosis in Breast and Colon Cancer Cells

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Abstract. *Aim: To explore the effects of PCK3145 beyond prostate cancer. Materials and Methods: Using Trypan blue, MTT proliferation assays, cell cycle and apoptosis analysis, we assessed the effects of PCK3145 on prostate (PC-3), breast (MCF-7) and colon (HT-29) human cancer cell lines and in osteosarcoma (MG-63) cells; any synergistic effects with docetaxel and oxaliplatin were also explored. Results: PCK3145 inhibited proliferation and induced apoptosis of PC-3, MCF-7 and HT-29 cells in a dose- and time-dependent manner but not in the MG-63 cell line, consistent with the low expression of the laminin receptor (LR) in the latter cell line. PCK3145 produced rapid (within 5min) and transient (up to 60 min) activation of MEK and ERK1/2. Synergistic effects were observed with docetaxel and oxaliplatin. Conclusion: PCK3145 can exert anticancer activity not only on prostate but also on breast and colon cancer cells, possibly through LR-mediated activation of MEK and ERK1/2 phosphorylation.*

Prostate cancer is the second most common cancer in men worldwide and the fifth leading cause of death from cancer in men. With an estimated incidence of 1.1 million men and 307,000 deaths in 2012, it accounts for 15% of the cancers diagnosed in men and 6.6% of the total deaths of cancer (1). Prostate secretory protein 94 (PSP94), also known as prostatic inhibiting peptide or β-microseminoprotein, was initially discovered as an hormone that reduced the levels of circulating follicle-stimulating hormone (2). PSP94 is one of the three predominant proteins secreted by the prostate gland along with prostate-specific antigen and prostatic acid phosphatase (3); it is also found in large quantities in the seminal fluid (4), in mucous glands and secretions (5), as well as in human female reproductive tissues, breast and in endometrial cancer cell lines (6). PSP94 expression has been shown to be dependant on the stage of prostate cancer; it is high in the normal prostate but progressively decreases as prostate cancer advances, with a complete loss of PSP94 expression in highly advanced prostate cancer (7). This down-regulation of PSP94 in prostate tumor tissues may be associated with a possible cancer survival mechanism (8, 9). Furthermore, a common genetic variant (rs10993994) in the 5' region of the gene encoding β-microseminoprotein (*MSMB*) was shown to be associated with circulating levels of PSP94 and prostate cancer risk (10). More recently, it was shown that men with low blood levels of PSP94 have increased risk of prostate cancer, regardless of race and ethnicity or rs10993994 genotype (11). It was suggested that PSP94 plays a protective role in prostate carcinogenesis; although the mechanisms for such protection are unclear, it was postulated that this may be achieved through tumor suppression as PSP94 has been observed to inhibit cell or tumor growth in *in vitro* and *in vivo* models (12) or by prevention or limitation of an intracellular fungal infection in the prostate (13).

Shukeir *et al*. examined 12 different synthetic peptide fragments that compose the full length of PSP94 in order to identify the fragment that was able to inhibit prostate cancer growth *in vitro* as PSP94; they found that a peptide formed by the amino acids 31-45 of the PSP94 sequence (which was called PCK3145) had the best antiproliferative and antimetastatic action in the *in vitro* and *in vivo* prostate cancer models tested and that equimolar concentrations of PSP94 and PCK3145 were similarly effective (14). The mechanism of action of PCK3145 is still not fully elucidated; PCK3145 was shown to reduce the levels of matrix metalloproteinase 9 (MMP-9) in animals (15), it has a CD44 cell surface recognition and internalization and also interferes with laminin signaling (16, 17). More recently, it was shown by another

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group that PCK3145 suppresses chemoresistant ovarian cancer cell and tumor growth *in vitro* and *in vivo*, possibly by modulating the Lin28b/Let-7 signaling pathway (18).

Herein, we investigated the action of PCK3145 on human MCF-7 breast cancer, HT-29 colon cancer and MG-63 osteosarcoma cell lines; the study of PCK3145 in prostate PC-3 cells was also included for reference purposes. Thus, we evaluated laminin receptor (LR) expression into four cancer cell lines and examined the anti-proliferative and apoptotic effect of PCK3145 on PC-3, MG-63, MCF-7 and HT-29 cells, as well as any additive or synergistic anticancer effect in combination with docetaxel or oxaliplatin.

Materials and Methods

Cell cultures. The PC-3 and MG-63 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). The MCF-7 breast cancer cell line was cultured in Eagle's Minimum Essential Medium (EMEM) (Gibco, Invitrogen) supplemented with 10% FBS. Human colorectal cancer cells HT29 were cultured in McCoy's 5A medium (Gibco, Invitrogen) supplemented with 10% FBS. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37˚C in a humidified atmosphere of 5% CO_2.

Treatments. All cell lines were grown up to 70-80% confluence in complete media. Twenty-four hours after seeding, cell media was changed to 5% FBS for another 24-h period. Then, the cells were treated with PCK3145 in various concentrations (5, 50, 500 nM and 5 μM) or docetaxel (1, 10 and 50 nM) for 48 and 96 h. For HT-29 cells, we used oxaliplatin (up to 15 nM) instead of doxetacel. Finally, treatments of PCK3145 together with docetaxel or oxaliplatin were used for 48 and 96 h in order to study a possible additive/synergistic effect.

Cell proliferation. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA). Cells were plated in 96-well plates at a cell density of 2,000 cells/well. After 24 h, the media was changed to 5% FBS for another 24 h, then cells were treated for 48 and 96 h with the appropriate drug or drug combination. Ten percent of MTT (5mg/ml) was added to each well in a humified atmosphere (37˚C, 5% CO_2). After 4h, plates were aspirated, 150 ml/well of DMSO was added and optical density measured at 570 nm (Versa max, Molecular Devices, Sunnyvale, CA, USA) as described previously (9, 19). MMT assays measure mainly the metabolic activity of cells in culture, indirectly associated with cell viability status. The concentration of drug used for further studies was the one that was able to reduce cell viability by 50%. For Trypan blue assays, cells were plated at a cell density of 3.5×10^4 cell/well in 6-well plates and exposed to PCK3145 in dose-dependent and time-dependent manner and cell numbers were counted as described previously (19).

Flow cytometry. Cells were plated at a cell density of 4×10^4 cells/well in 24-well plates and grown in DMEM containing 10% FBS. Twenty-four hours after plating, the medium was changed to 5% FBS, the cells were exposed to PCK3145 (5 μM), or docetaxel (50 nM), or oxaliplatin (15 nM), as well as to a combination of PCK (5 μM) and docetaxel (50 nM) or oxaliplatin (15 nM). Forty-eight and 96 h after treatment, the adherent and floating cells were combined and analyzed using the TACS Annexin V-FITCH Apoptosis detection kit (Trevigen Inc, Gaithersburg, MD, USA). Cells were analyzed using CyFlow ML (Partec, Münster, Germany) as described previously (19).

Cell cycle analysis. For cell cycle analysis, cells were exposed for 48 and 96 h at 37˚C in the appropriate media supplemented with 5% FBS in 24-well plates at a cell density of 50,000 cells/well with and without the agent or the combination of drugs. Adherent and floating cells were combined, washed with PBS and fixed overnight at 4˚C in 70% ethanol. Fixed cells then were stained with CyStain DNA 1step (05-5004) (Partec, Münster, Germany). DNA content was analyzed on a FACS Calibur CyFlow ML flow cytometer (Partec, Münster, Germany) using the ModFit software [Flowmax3.0 Software (1997-2007) Version 3.0(b4), Sunnyvale, CA, USA].

Western blot analysis. Cells were seeded in 6-well plates and grown in the appropriate media containing 10% FBS. Twenty-four hours prior protein extraction, the growth medium was changed to 5% FBS. The cell extracts were obtained by lysis of the cells in RIPA buffer (50 mM Tris-HCl; 150 mM NaCl; Sigma) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Cell Signaling, Beverly, MA, USA). After a 30 min incubation on ice, the lysates were cleared by centrifugation (14,000 rpm, 30 min and 4˚C). Protein concentrations were determined by the Bio-Rad protein assay (BIO-RAD Laboratories, Hercules, CA, USA). Equal amount of cell lysates (20 μg) were heated at 95˚C for 5 min, electrophoresed on 12% SDS-PAGE under denaturing conditions and transferred onto nitrocellulose membrane (BIO-RAD Laboratories). The membranes were probed overnight with primary antibodies against LR (sc-20979, c: H-141), (Santa Cruz, TX, USA), phospho-ERK1/2, total ERK1/2, phospho-MEK1/2 and phospho-c-Raf (Cell Signaling), all at 1:1,000 dilution in TBS/T containing 5% BSA and GAPDH (Santa Cruz Biotechnology). The blots were incubated with a secondary goat antibody raised against rabbit IgG conjugated to horseradish peroxidase (1:2,000 dilution) (sc2004) (Santa Cruz Biotechnology). The bands were visualized by exposure of the blots to X-ray film after incubation with freshly made ECL substrate for 5 min (SuperSignal, Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis. The data of triplicate experiments were compared. Values are means±standard deviation (SD). Statistical analysis (Student's *t*-test; statistical significance set at $p<0.05$) was performed in the results of PCK3145 only. Its apoptotic effects were compared *vs*. control and the apoptotic effects of the combined treatment of PCK3145 with docetaxel or oxaliplatin were compared with those of PCK3145 alone. The effects of PCK3145 in combination with docetaxel or oxaliplatin on cell cycle were compared against the corresponding effects of docetaxel or oxaliplatin alone.

Results

Expression of laminin receptor (LR) in cancer cell lines. Western blot (WB) analysis confirmed the presence of LR (37 kDa precursor, LRP) in proteins extracted from PC-3, MCF-7, HT-29 cancer cells (Figure 1A). In a subsequent

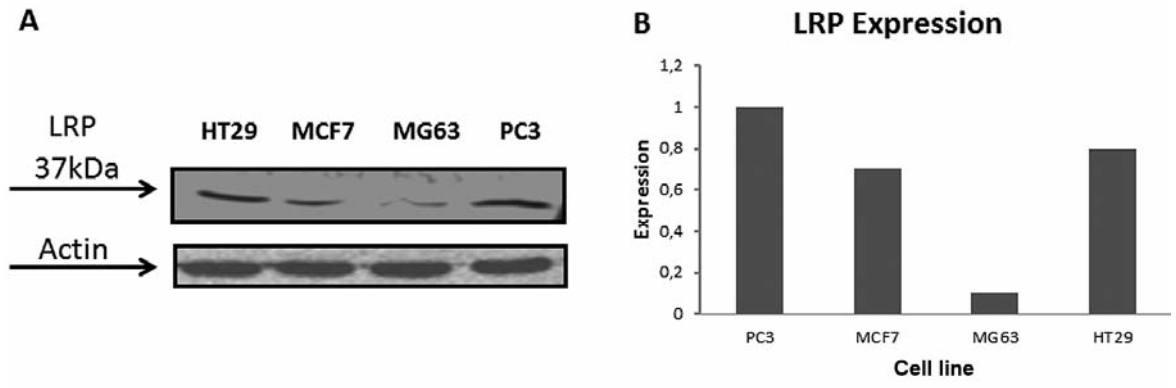

Figure 1. *Expression of laminin receptor (LR) in human prostate (PC-3) breast (MCF-7) and colon (HT-29) cancer and osteosarcoma (MG-63) cell lines. (A): Expression of the 37-kDa laminin receptor precursor (LRP) in the PC-3, MCF-7, MG-63 and HT-29 cancer cell lines. (B): Densitometric analysis of LR expression in MCF-7, MG-63 and in HT-29 cells, relative to the PC-3 cancer cells expression (arbitrarily set to 1).*

densitometric analysis of the WB signals, the MG-63 osteoblast-like cells seemed to have the lowest expression of LR protein among all cell lines (about 10%), whilst that in the MCF-7 breast cancer cells was about 70% and that in the HT-29 colon cancer cells was about 80%, considering LR expression in the PC-3 cancer cells as 100% (Figure 1B).

Effects of PCK3145, docetaxel and oxaliplatin, used as single agents, on cell proliferation. Increasing concentrations of PCK3145 (5 nM, 50 nM, 500 nM, 5 μM) resulted in a dose-dependent inhibition of the PC-3 cells metabolism/growth, as assessed by the MTT assay, with a 50% inhibition evident at concentrations of 5 μM of PCK3145 after incubation for 48 or 96 h (data not shown). PCK3145 was also found to inhibit metabolism/growth of the MCF-7 and HT-29 but not of the MG-63 cell line (data not shown). The effects of PCK3145 on the growth of the same cell lines were also studied by Trypan blue exclusion assays measuring the number of living and dead cells. The results in Figure 2A show that the effect of different doses of PCK3145 at 48 h were consistent with those obtained using the MTT assay. Since no effects of PCK3145 were found on the growth of the MG-63 cells, this cell line was not used for further investigation in the present study.

In the MMT proliferation assays, docetaxel (used in doses up to 50 nM) also produced a dose-dependent cell metabolism/growth inhibition in the PC-3, MCF-7 but also in the MG-63 cell line, while oxaliplatin (used in doses up to 15 nM) showed an effect in the HT-29 cell line where it was tested as control of cell death (data not shown). These effects were consistent with the findings of the Trypan blue exclusion assays for these compounds; Figure 2B and 2C show the effects of various doses of docetaxel and oxaliplatin, respectively, at 48 h.

Effects of PCK3145, docetaxel and oxaliplatin, used as single agents and as a combination, on apoptosis and cell death. PCK3145 (5 μM) showed a statistically significant ($p<0.05$) apoptotic effect an all three cancer cell lines (PC-3, MCF-7 and HT-29) tested at both 48 and 96 h (Figure 3A). Docetaxel (50 nM) and oxaliplatin (15 nM) also showed an apoptotic effect similar to that of PCK3145 in the cell lines tested (PC-3 and MCF-7 for docetaxel and HT-29 for oxaliplatin (Figure 3B and 3C).

Combination treatment using docetaxel (50nM) and PCK3145 (5 μM) seemed to increase the PCK3145-induced apoptosis of PC-3 cells after 48 h of co-treatment, although the result did not reach statistical significance probably due to the variability in results of the PCK3145-alone treatment in this case; the effect seemed not to increase further after 96 h of co-treatment (Figure 3A). In the MCF-7 cell line, however, the combination of the same doses of PCK3145 and docetaxel showed a statistically significant increase in apoptosis after 48 h of co-treatment, which seemed to slightly increase further after 96 h (Figure 3B). Similarly, a statistically significant synergistic effect was produced by the co-treatment of HT-29 cells with 15 nM oxaliplatin and 5 μM PCK3145 for 48 h, which seemed not to increase further after 96 h of co-treatment (Figure 3C).

Cell-cycle analysis (Table I) did not produce evidence of cell-cycle arrest of any cells checked into a cell-cycle phase by PCK3145. However, docetaxel, as expected, showed an increased percentage of distribution of PC-3 and MCF-7 cells into the G_2/M phase, which was statistically significantly reduced ($p<0.05$) in the combination treatment with PCK3145 with docetaxel (Table I). Oxaliplatin also showed an increased percentage of distribution of HT-29 cells into the G_2/M phase as expected, which was slightly further increased in the combination treatment with

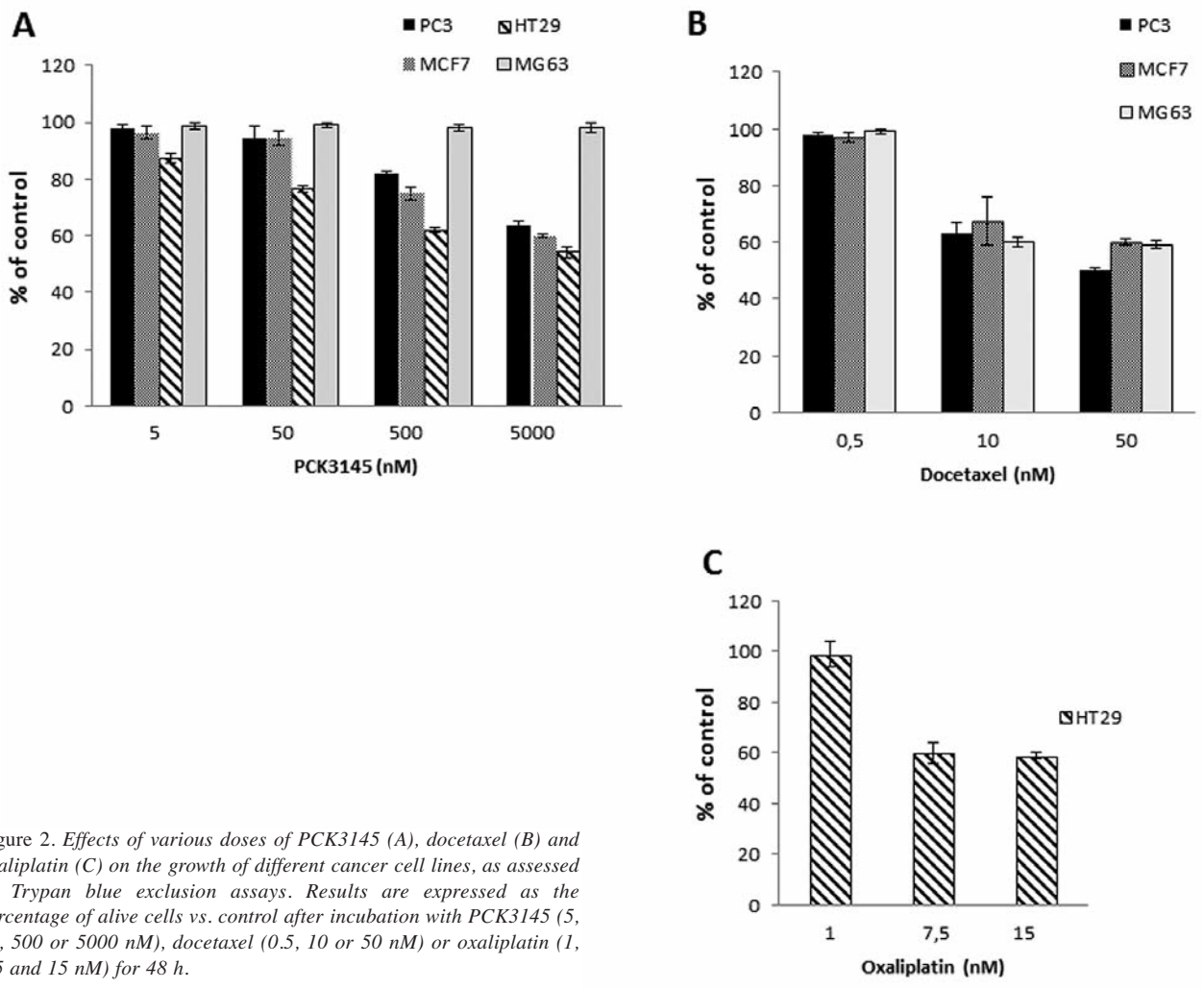

Figure 2. *Effects of various doses of PCK3145 (A), docetaxel (B) and oxaliplatin (C) on the growth of different cancer cell lines, as assessed by Trypan blue exclusion assays. Results are expressed as the percentage of alive cells vs. control after incubation with PCK3145 (5, 50, 500 or 5000 nM), docetaxel (0.5, 10 or 50 nM) or oxaliplatin (1, 7.5 and 15 nM) for 48 h.*

Table I. *Distribution (%±SD) of living PC-3, MCF-7 and HT-29 cells into the phases of the cell cycle (Gap 0/Gap1, Synthesis and Gap2/Mitosis), before (control) and after exposure to PCK3145 (5 μM), docetaxel (50 nM) and oxaliplatin (15 nM) alone, as well as PCK3145-plus-docetaxel and PCK3145-plus-oxaliplatin, as assessed by flow cytometry.*

Cell line	Treatment	G_0/G_1	S	G_2/M
PC3	Control	52.66±0.35	25.92±0.67	21.42±3.18
	PCK3145	54.11±4.53	22.61±1.42	23.28±2.26
	Docetaxel	17.76±3.62	17.91±0.77	64.34±0.45
	Docetaxel+PCK3145	35.92±0.15	18.32±0.16	45.76±0.81*
MCF7	Control	58.83±0.23	20.56±0.44	20.61±0.33
	PCK-3145	56.32±2.53	19.76±0.42	23.92±1.99
	Docetaxel	12.23±0.73	9.13±0.85	76.64±0.45
	Docetaxel+PCK3145	30.88±0.23	12.66±0.45	56.45±0.75*
HT29	Control	49.73±2.25	28.85±0.60	21.42±0.54
	PCK3145	49.65±1.32	25.38±0.71	24.97±0.98
	Oxaliplatin	38.16±1.25	3.88±1.44	57.96±1.69
	Oxaliplatin+PCK3145	39.65±0.24	2.06±0.82	68.29±2.32

Statistical comparisons of the combination treatments were made *vs*. the corresponding docetaxel or oxaliplatin treatments alone; *p<0.05.

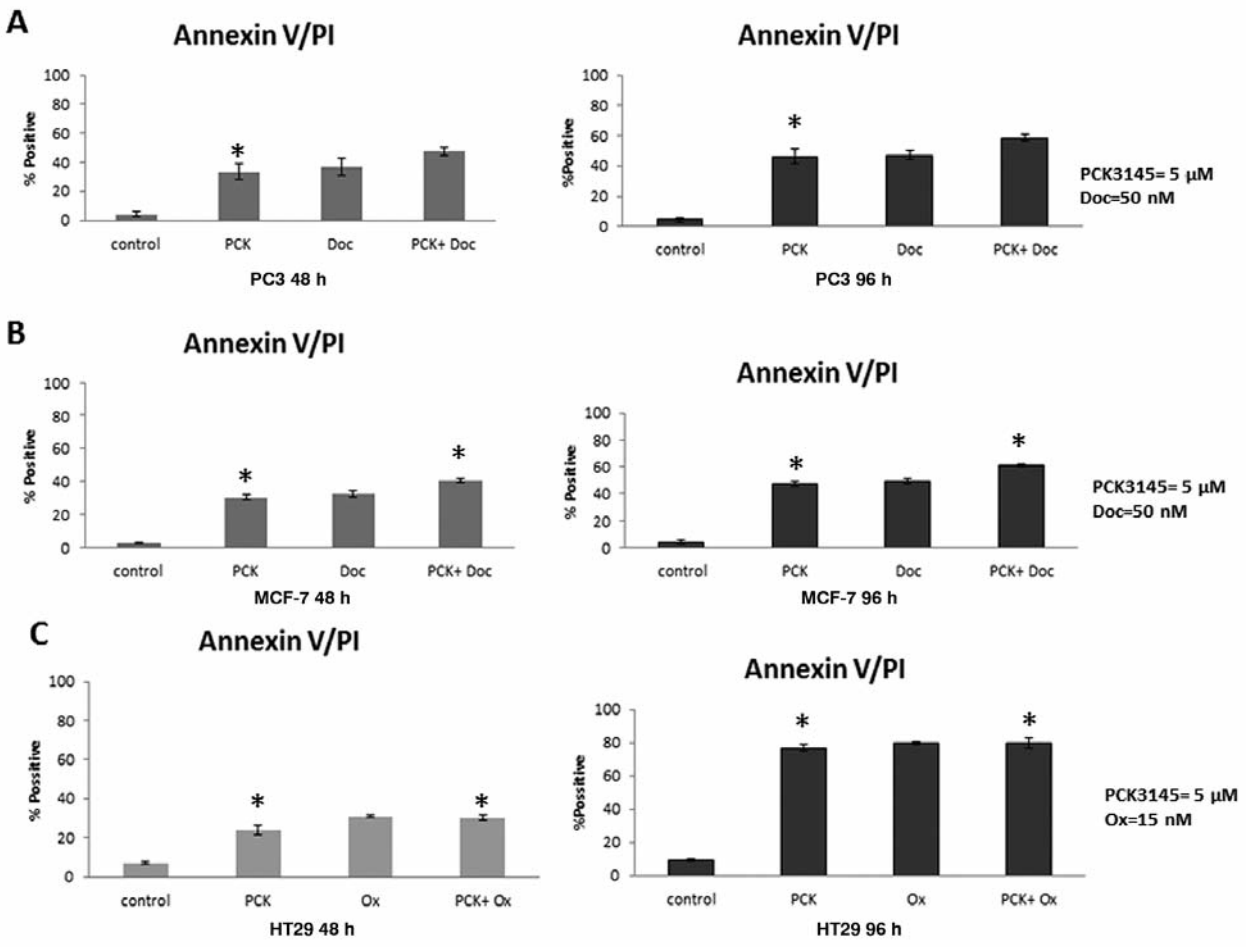

Figure 3. *Apoptotic effects of PCK3145 (5 µM), docetaxel (50 nM) and oxaliplatin (15 nM) used alone or in combination on PC-3 (A), MCF-7 (B) and HT-29 (C) cancer cell lines. Apoptosis data were obtained by flow cytometry in triplicate experiments and are expressed as number of apoptotic cells (% positive cells) compared to the total cell number after 48 h (left panels) and 96 h (right panels) of treatment. Statistical comparisons for PCK3145 were made vs. control (when PCK3145 was used alone) and vs. PCK3145 alone in the combination treatments. *p<0.05.*

PCK3145 with oxaliplatin without, however, reaching statistical significance (p=0.062) (Table I).

Effects of PCK3145 on ERK1/2 signaling. We investigated the possible activation of ERK1/2 signal transduction pathway by PCK3145. Our experiments involved incubation of PC-3, MCF-7 and HT-29 cells with 5 µM PCK3145 for 0, 5, 15, 30 and 60 min. PCK3145 activated ERK1/2 phosphorylation within 5 min, while, after 1 h of incubation with PCK3145, the ERK1/2 phopshorylation returned at control levels (Figure 4A). PCK3145 produced a 6-fold increase in the phosphorylation of ERK1/2 in PC-3 cells, a 5.8-fold increase in MCF-7 cells and a 3-fold increase in HT-29 cells after 5 min of incubation, which appeared to further increase at 15 min (Figure 4B). However, a different pattern of phosphorylation was noticeable in MCF-7 and HT-29, where the maximum effect on ERK1/2 phosphorylation was at 5 min and then gradually decreased. The study of this signaling pathway upstream ERK1/2 in PC-3 cells revealed that 5 min of PCK3145 incubation resulted in phosphorylation of c-Raf, as well as of MEK1/2 (Figure 4C).

Discussion

Among different peptide fragments encompassing the whole PSP94 sequence, PCK3145 was selected for further study as it exhibited the most promising *in vitro* and animal *in vivo* results in prostate cancer (12, 14). Its activity is consistent with the biological activity of PSP94, the reduced levels of PSP94 in prostate cancer, as well as with the association of PSP94 with the etiology and risk in prostate cancer (13). It has been known in the literature that PSP94 expression is not

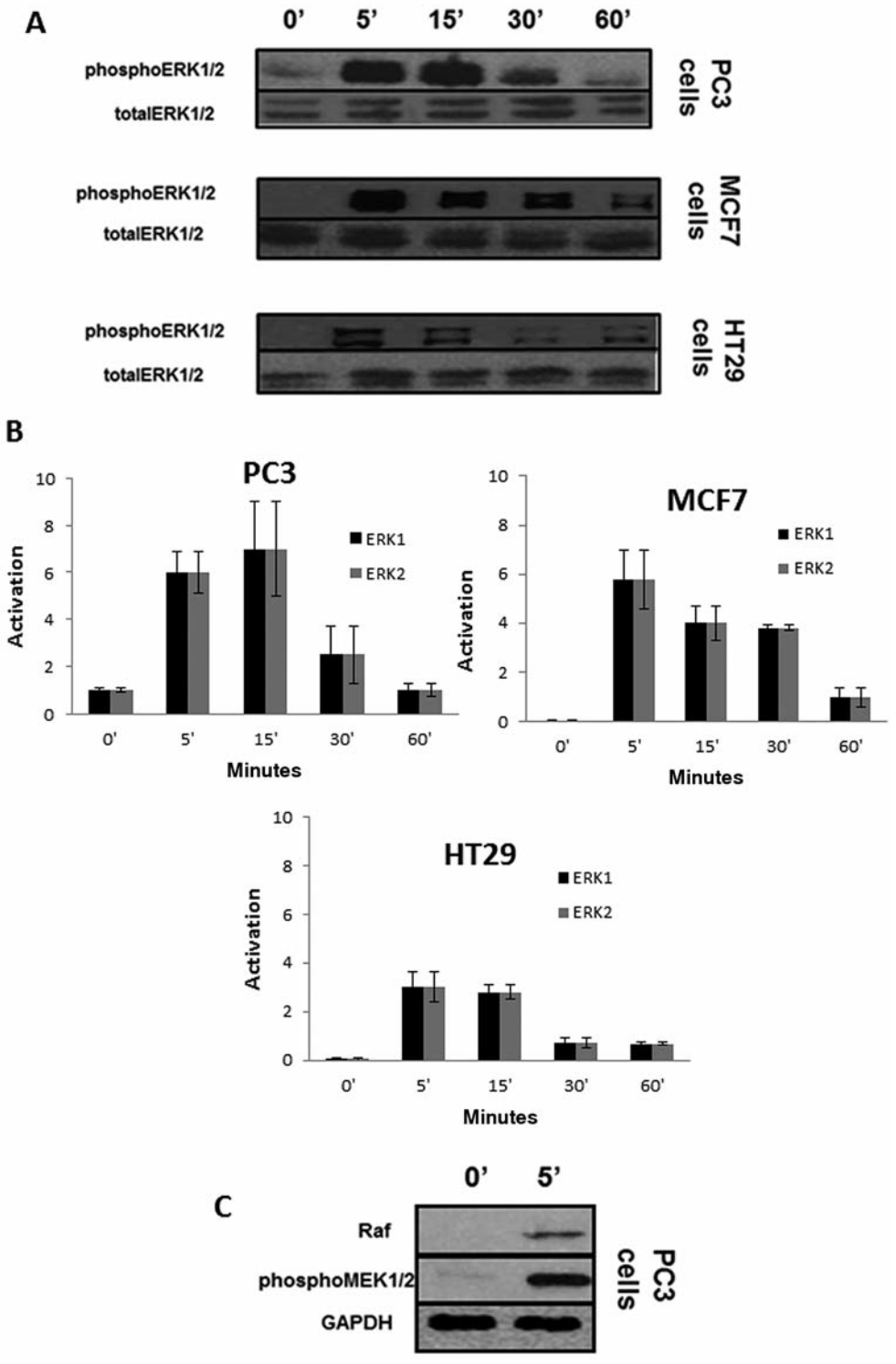

Figure 4. *Effects of PCK3145 in ERK1/2 signaling. (A) Western blot analysis of the ERK1/2 phosporylation after incubation PC-3, MCF-7 and HT-29 cells (cultured with 0.5% FBS) with PCK3145 (5 μM) for 5, 15, 30 and 60 min. (B) Densitometry analysis of immunoblots revealed that PCK3145 activated ERK1/2 phosphorylation on PC-3 cells by 6-fold after 5 min, by 7-fold after 15 min by 2.5-fold at 30 min and subsequently decreased to control levels after 60 min of incubation. In MCF-7 and HT-29 cells the maximum phosphorylation was observed at 5 min (5.8-fold for MCF-7 and 3-fold for HT-29) and, subsequently, the activation gradually decreased. (C) Immunoblot analysis showed activation for MEK1/2 and c-Raf on PC-3 cells after 5 min of PCK3145 (5 μM) administration.*

prostate-specific and its expression was also reported in human female reproductive tissues, breast and in endometrial cancer cell lines (6). More recent studies revealed that PSP94 expression is decreased in patients with chemoresistant ovarian cancer and that PCK3145 selectively suppresses growth of ovarian cancer cells *in vitro* and *in vivo* (18).

The mechanism of action of PCK3145 in *in vitro* and *in vivo* prostate cancer models has not been fully elucidated yet; it has been suggested that the inhibition of MMP-9 secretion caused by PCK3145 requires cell surface LR signaling and in particular the 37-kDa laminin receptor precursor (37LRP) (17). LR signaling has been shown to involve kinase/phosphatase cascades in various models (20). The LRP/LR is over-expressed in many types of cancer, including breast, colorectal, ovarian and prostate cancerous tissues, as well as lymphomas, and shown to have a vital role in growth and metastasis of tumor cells, angiogenesis and resistance to chemotherapy (21, 22). Anti-LRP/LR-specific antibodies reduce adhesion and invasion of breast and esophageal, as well as metastatic liver, lung, cervix, colon and prostate cancer cells and inhibit angiogenesis *in vitro* (23-26).

Herein, we documented that PCK3145 exerted anticancer actions on other cancer cell types, such as MCF-7 breast cancer cells and HT-29 colon cancer cells, an action that correlated positively with evidence of high LR expression in these cells and was consistent with PCK3145's activity on the LR-high expressing PC-3 prostate cancer cells (considered as the positive control in our study). The data obtained were corroborated by the absence of any anticancer action of PCK3145 on the LR-low expressing MG-63 osteosarcoma cells. Such mechanism, that involves LR, was shown to activate the MEK and ERK1/2 pathways in this study.

To define whether PCK3145 and chemotherapy may have an additive or synergistic effect on cancer cell lines, we first investigated if the combinational treatment could increase the rate of apoptosis compared to the effects of each anticancer agent separately. We witnessed a synergistic effect by using the combination of docetaxel (50 nM) and oxaliplatin (15 nM) plus 5 μM PCK3145 on MCF-7 and HT-29 cells, respectively.

In conclusion, the data reported here suggest for the first time that PCK3145 can have general anticancer actions on a wide type of cancer cells (other than prostate), which express high levels of LR, implicating molecular mechanisms present downstream the ERK1/2 activation. Furthermore, the combination of PCK3145 with docetaxel or oxaliplatin is likely to be beneficial.

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