The Steroidal Na^+/K^+ ATPase Inhibitor 3-[(*R*)-3-Pyrrolidinyl]oxime Derivative (3-R-POD) Induces Potent Pro-Apoptotic Responses in Colonic Tumor Cells

SAAD HUSSIN ALKAHTANI

Department of Zoology, Science College, King Saud University, Riyadh, Saudi Arabia

Abstract. *Recently, potent anticancer actions of the steroidal Na^+/K^+ ATPase inhibitor 3-[(R)-3-pyrrolidinyl]oxime derivative 3 (3-R-POD) have been reported for multiple cell lines, including prostate and lung cancer cells. In the present study, the anticancer action of 3-R-POD was addressed in colonic tumor cells. Treatment of Caco2 colonic tumor cells with increasing concentrations of 3-R-POD induced potent, dose-dependent inhibition of cell growth as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In addition, the APOpercentage apoptosis assay revealed significant pro-apoptotic responses, suggesting that the anticancer activity of this steroidal Na^+/K^+ ATPase inhibitor in colonic tumors takes places mainly through the induction of strong pro-apoptotic effects. Focussing on the molecular mechanism that may regulate these interactions, 3-R-POD was shown to induce significant early actin re-organization and late Protein Kinase B (AKT) de-phosphorylation. Finally, the 3-R-POD-induced inhibition of cell growth and early actin reorganization in colonic cancer cells remained unchanged when cells were pre-treated with pertussis toxin, thus excluding possible interactions of this inhibitor with G-coupled receptors. These results indicate that 3-R-POD induces potent pro-apoptotic responses in colonic tumor cells governed by actin re-organization and inhibition of AKT pro-survival signaling.*

In a recent study, we synthesized and characterized 17 steroidal Na^+/K^+ ATPase inhibitors (1). These agents were tested for their antitumor activity in a pre-screening analysis using various National Cancer Institute, USA, cancer cell lines. The most potent inhibitor identified in the *in vitro* study, the 3-[(*R*)-3-pyrrolidinyl]oxime derivative 3 (3-R-POD), was shown to be highly effective both *in vitro* in a large number of cancer cell lines, including PC3- and DU145 (prostate), A549 (lung) SF-268 (CNS) and CaCi (renal) cancer cells, as well as *in vivo* in anticancer efficacy studies in prostate and non-small cell lung cancer animal models (1). These results revealed significant tumor-growth inhibition activity of this compound in prostate and lung cancer cells. However, the potential effects of this compound in colonic tumor cells remained unknown. In the present study, the *in vitro* efficacy of 3-R-POD on the cell fate of colonic cancer cells was addressed. By using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and APOpercentage apoptosis assays, cell growth and apoptotic cell death were analyzed. To gain molecular insights, actin cytoskeleton re-organization and AKT pro-survival signaling were evaluated. Finally, by using pertussis toxin, the possible cross-talk of this inhibitor with G-protein coupled receptors (GPCRs) was addressed.

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Correspondence to: Saad Hussin Alkahtani, Ph.D., Department of Zoology, Science College King Saud University, P.O. Box 271942, Riyadh 11352, Saudi Arabia. Tel: +96 6505233673, e-mail: salkahtani@ksu.edu.sa

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Materials and Methods

Cell culture. The Caco2 human colon cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and was adapted to grow in RPMI-1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 5-10% fetal bovine serum and antibiotics in a humidified atmosphere with 5% CO_2 at 37˚C. Based on previous titration experiments (1) different concentrations (100 nM to 10 μM) of 3-R-POD, kindly provided by Dr. T. Calogeropoulou (National Hellenic Research Foundation, Athens, Greece) was used throughout this study. The synthesis of this steroidal Na^+/K^+ ATPase inhibitor has been described in detail elsewhere (1).

MTT assay. Cell proliferation/viability was assessed by MTT assays (Sigma, St. Louis, MO, USA). Cells were cultured in 96-well plates for 24 h (7-10,000 cells/well) and were subsequently incubated with three different concentrations (in triplicates) of the drug (ranging from 100 nM-10 μM) in the presence or absence of pertussis toxin

(100 ng/ml; Biomol Research Laboratories, Pennsylvania, PA, USA) in serum-containing medium for 48 h. Serum-free conditions were used as a control. At the end of the incubation, the medium was aspired and MTT dissolved in RPMI-1640 was added to a final concentration of 0.25 mg/ml. After 4 hours' incubation (37˚C, 5% CO_2, dark), the supernatant was discarded, the converted dye was solubilized by adding 200 μl dimethyl sulfoxide (DMSO) and the absorbance was measured at 550 nm with reference at 655 nm using a spectrophotometer.

APOPercentage apoptosis assay. Caco2 cells were cultured in 96-well plates for the APOPercentage apoptosis assay (Biocolor Ltd., Belfast, Ireland). They were stimulated with 100 nM 3-R-POD for 24 and 48 h in serum-containing medium. Apoptosis was calculated as a percentage that of untreated cells.

Measurement of G/total actin ratio by Triton X-100 fractionation. The Triton X-100 soluble G-actin and total-actin containing fractions of cells exposed to 10 μM of 3-R-POD in the presence (1 h pre-treatment) or absence of 100 ng/ml of pertussis toxin (Biomol Research Laboratories) were prepared as previously described (2, 3). A decrease of the Triton X-100-soluble (G-) to the total (T-) actin ratio is indicative of actin polymerization.

Immunoprecipitation and western blotting. Cells were incubated with 100 nM 3-R-POD for the indicated time periods shown in Figure 4A, washed twice with ice-cold PBS and suspended in 500 μl ice-cold lysis buffer (50mM Tris/HCl, 1% Triton X-100 pH 7.4, 1% sodium deoxycholate, 0.1% Sodium dodecyl sulfate (SDS), 0.15% NaCl, 1 mM EDTA, 1 mM sodium orthovanadate) containing protease inhibitor cocktail (Sigma). The protein concentration was determined using the Bradford assay (BioRad, Ireland, UK). Sixty micrograms of protein were solubilized in sample buffer at 95˚C for 5 min and resolved by 10% SDS-Polyacrylamide gel electrophoresis (PAGE). For immunoblotting, proteins were electro-transferred onto a PVDF membrane and blocked with 5% nonfat milk in Tris-buffered saline TBS-0.10% Tween 20 (TBST) at room temperature for 1 hour. The membrane was then incubated with phospho-AKT (Thr308) (1:100; Santa Cruz Biotechnology, California, USA) at 4˚C overnight. After washing with Phosphate Buffered Saline with Tween-20 PBST and subsequent blocking, the blot was incubated with secondary anti-rabbit antibody (1:5,000; GE Healthcare, Louis, USA) for 1 h at room temperature. After washing, antibody binding was detected with the ECL detection reagent (Amersham, Buckinghamshire. Germany). For controls, the blots were stripped in stripping buffer (Carl Roth, Heidelberg, Germany) at 56˚C for 30 min. After washing with TBST and subsequent blocking, the blot was incubated with secondary anti-rabbit antibody (1:2,000; Cell Signaling, New Jersey, USA) for 1 h at room temperature. Antibody binding was detected with the ECL detection reagent (Amersham) according to the manufacturer's instructions.

Statistical analysis. Data are provided as means±SEM, n represents the number of independent experiments. Data were tested for significance using unpaired Student's *t*-test or ANOVA as appropriate. Differences were considered statistically significant when *p*-values were less than 0.05. Statistical analysis was performed with GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA; www.graphpad.com)

Results

The *in vitro* anticancer activity of 3-R-POD towards Caco2 colon cancer cells was initially assessed by the MTT cell viability assay. Figure 1 shows the strong dose-dependent potency of this compound in the range of 100 nM to 10 μM after 48 h incubation. To further analyze the anticancer efficacy, the pro-apoptotic effects of this compound was addressed in the Caco2 colon cancer cell line by using the APOPercentage apoptosis assay. Figure 2 depicts significant induction of apoptosis after 24 and 48 hours' incubation of the cells with 100 nM 3-R-POD.

To further gain insights into the molecular mechanism implicated in the 3-R-POD-initiated cell growth arrest in colonic tumor cells, the involvement of actin cytoskeleton reorganization was assessed. Indeed, early actin restructuring has been shown in the past to be implicated in initiating apoptotic responses (4). Accordingly, Caco2 cells were incubated with 10 mM 3-R-POD for 15 and 60 min and actin polymerization was quantitatively determined as described in detail elsewhere (3). As shown in Figure 3, a significant decrease of the G/F-actin ratio was evident, pointing to important actin polymerization. This finding implies rapid actin cytoskeleton reorganization upon 3-R-POD treatment and is in line with the observed induction of the pro-apoptotic response of Caco2 colonic tumor cells.

The 3-R-POD-induced cell growth arrest manifested in colonic tumor cells was associated with considerable down-regulation of the activity of the pro-survival kinase AKT. As shown in Figure 4, AKT phosphorylation at Thr-308 significantly decreased after 12 hours' incubation of the cells with the compound, indicating that this inhibitor blocks the focal adhesion kinase PI3k/AKT pro-survival signaling pathway.

Finally, to address the possible cross-talk of 3-R-POD on GPCRs, its effects on cell growth arrest and actin reorganization were measured in the presence of pertussis toxin. As shown in Figures 1B and 3, both, MTT assay and G/F-actin ratio measurements revealed the effects of 3-R-POD to be unchanged in the presence of pertussis toxin. These results exclude possible interactions of 3-R-POD with GCPRs.

Discussion

The present study addressed the antitumor activity of 3-R-POD in colonic cancer cells. Incubation of Caco2 cells with this derivative exerted a) strong cell growth arrest as documented by MTT and APOPercentage apoptosis assays; b) potent actin cytoskeleton re-organization, as determined by quantitative actin polymerization measurements; and c) late inhibition of the pro-survival kinase AKT. These findings fully support the recently postulated strong antitumor activity of this compound towards prostate and lung cancer cells (1)

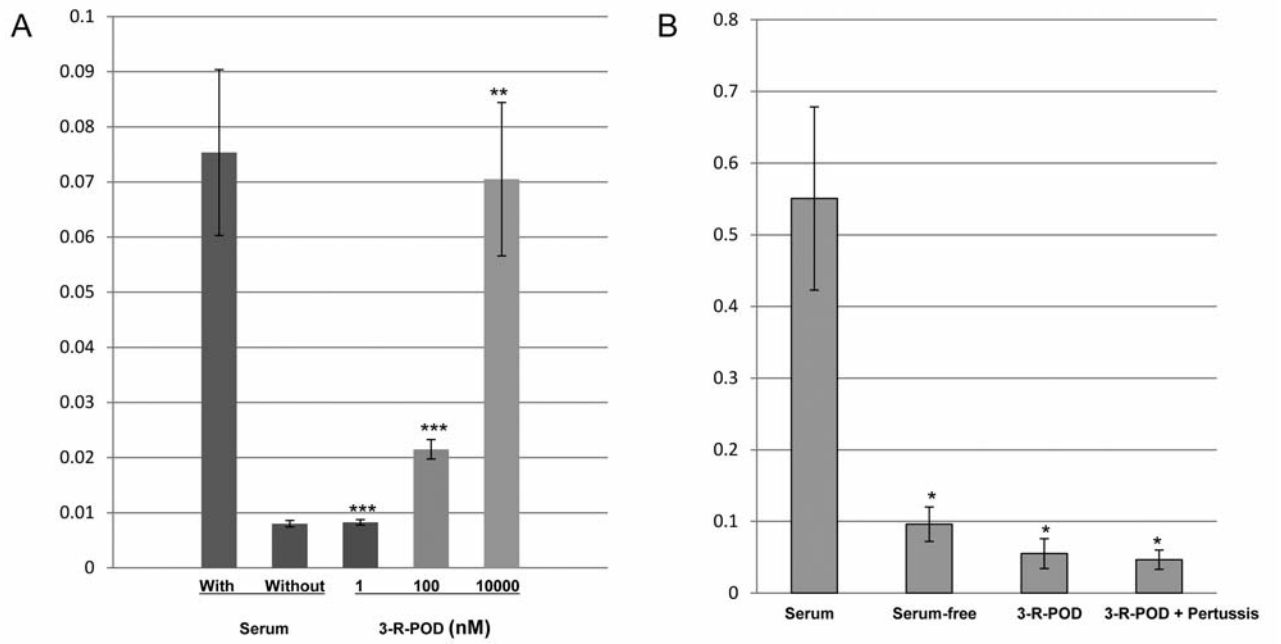

Figure 1. *Effects of 3-[(R)-3-pyrrolidinyl]oxime derivative 3-R-POD on viability of Caco2 cells treated with and without pertussis toxin. A: Methylthiazol Tetrazolium MTT assay showing the effects on cell viability of Caco2 cells treated for 48 h with increasing concentrations of 3-R-POD. Serum-free conditions were used as control. Bars represent mean values±SE from n=3 experiments in triplicate. Significantly different from untreated controls with serum at **p<0.01 and ***p<0.001. B: MTT assay showing the effects on cell viability of Caco2 cells treated for 48 h with 10 μM 3-R-POD in the presence of 100 ng/ml of pertussis toxin. Serum-free conditions were used as control. Bars represent mean values±SE from N=6 experiments in triplicate. Significantly different from untreated control with serum at *p<0.05.*

and argue that 3-R-POD may also be highly efficient in the inhibition of colonic tumor cell growth.

The sodium potassium pump (Na^+/K^+ ATPase) is a transmembrane protein that was recently linked to tumor treatment, since the alpha-1 subunit of this pump may represent a novel target for therapy of glioblastoma, melanoma and non-small cell lung cancer (5-7). In line with these reports, recent studies from our group demonstrated potent antitumor activity for a specific sodium potassium pump inhibitor, 3-R-POD, in prostatic and lung tumors, both *in vitro* and in animal studies (1).

The actin cytoskeleton has been recognized as an important regulatory system controlling various cell functions (4, 8-11). These studies clearly established a pivotal role of modified actin organization and signaling that may link various membrane-initiated actions to the regulation of key cellular outcomes in different cancer cell types (12-19). Recently, several studies postulated a key role of actin remodeling in apoptosis, cell death and aging (20-25). In line with these reports, early actin restructuring has also been implicated in initiating apoptotic responses in colonic (26, 27) and prostatic (28, 29) cancer cells and neuroblastoma (30), as well as in gastrointestinal epithelial cells (25), and other epithelial cells (24, 31). The results shown in the present study fully support this, since 3-R-POD, which

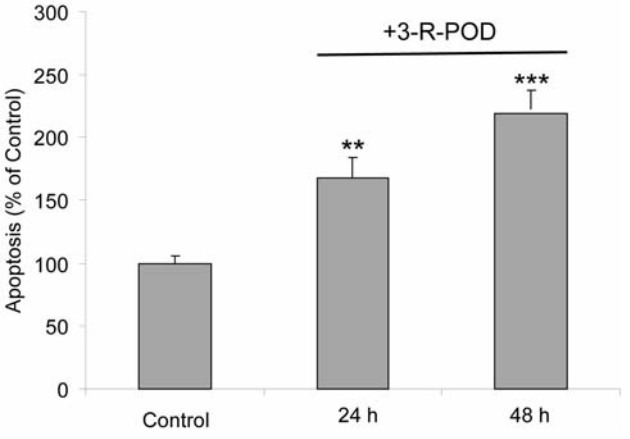

Figure 2. *Effects of 3-R-POD on apoptosis of Caco2 cells. APOPercentage apoptosis assay showing the apoptotic cell death of Caco2 cells treated for 24 or 48 h with 100 nM 3-R-POD. Bars represent mean values±SE as a percentage to that of the control (taken as 100), from n=4 experiments. Significantly different from untreated cells at **p<0.01 and ***p<0.001.*

was shown to provoke potent growth inhibition of colonic cancer cells, initially induced significant restructuring of actin cytoskeleton. Further studies are now required to provide the

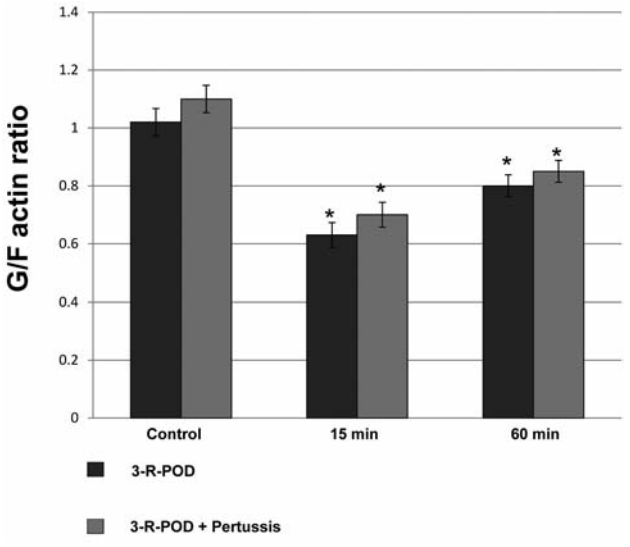

Figure 3. *Modulation of the dynamic equilibrium between globular G- and filamentous F-actin in 3-R-POD -stimulated Caco2 cells. Cells were stimulated with 10 μM 3-R-POD in the presence (red bars) or absence (blue bars) of 100 ng/ml of pertussis toxin for the indicated time. G- and F- actin were measured by quantitative immunoblot analysis after Triton X-100 subcellular fractionation. Bars present the mean G/F actin value±SE of n=3 independent duplicate experiments. Significantly different from untreated cells at *p<0.05.*

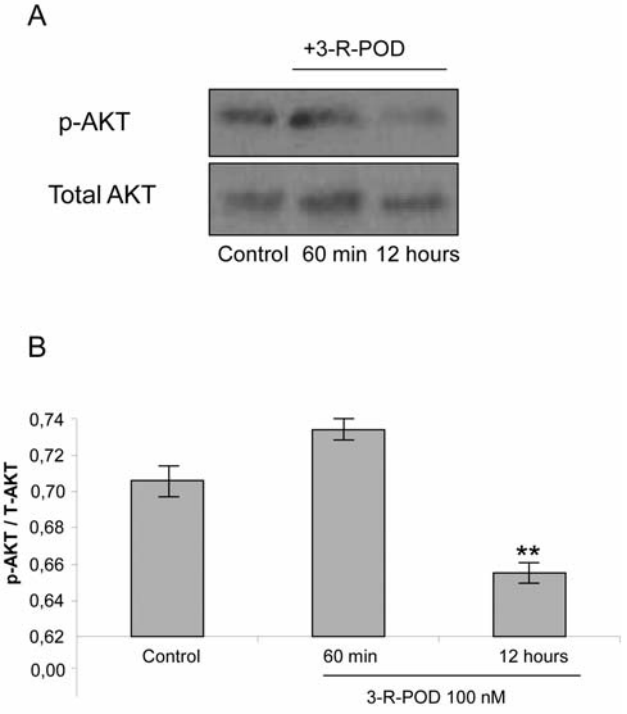

Figure 4. *Effects of 3-R-POD on AKT phosphorylation in Caco2 cells. Caco2 cells were stimulated with 100 nM 3-R-POD for the indicated time periods. Following cell lysis, equal amounts of total lysates were immunoblotted with a specific antibody against phospho-AKT and total AKT. Immunoblots were analyzed by densitometry. The intensity of phospho-AKT was normalized to the intensity of the corresponding total AKT band. Blots are from a representative experiment (A), and the relative fold increases (mean values±SE) from n=3 independent experiments of AKT phosphorylation with that of untreated **p<0.01.*

mechanistic link between early actin redistribution and the apoptotic responses in colonic tumor cells.

The inhibition of AKT phosphorylation, demonstrated after long-term incubation of the cells with 3-R-POD, indicate that this compound targets main pro-survival signaling pathways in colonic tumors. This suggests that such Na^+/K^+ ATPase inhibitors may interfere with central cell signaling events controlling tumor cell fate.

The specific inhibitor of the $Na^+/K^+/Cl^-$ co-transporter (NKCC1) furosemide, has been reported to inhibit phosphorylation of extracellular signal-regulated kinases ERK, induced by thrombin, a GPCR (32). In order to determine whether the Na^+/K^+ ATPase inhibitor 3-R-POD may also interact with GPCR s, experiments were performed in the presence of pertussis toxin, a wide-range GPCR inhibitor. As indicated by the MTT assay (Figure 1B) and the actin polymerization assessment (Figure 3), the 3-R-POD effects remained unchanged in the presence of the inhibitor, thus excluding possible cross-talk of this compound with GPCR.

In conclusion, the results presented here demonstrate for the first time, as far as we are aware of, potent antitumor responses to 3-R-POD in colonic tumor cells. These effects are mediated by rapid and significant actin re-organization and inhibition of the signaling kinase AKT, implicated in cell survival control. Taken together, the present results and the recently presented *in vitro* and *in vivo* studies with 3-R-POD (1) signify that this steroidal Na^+/K^+ ATPase inhibitor may represent a pharmacological candidate for colonic tumor drug development.

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