A Steroidal Na\(^+\)/K\(^+\) ATPase Inhibitor Triggers Pro-apoptotic Signaling and Induces Apoptosis in Prostate and Lung Tumor Cells

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Abstract: Recently we have reported potent anti-cancer actions of various steroidal Na\(^+\)/K\(^+\) ATPase inhibitors in multiple cell lines. Furthermore, the most powerful compound identified in this study, the 3-[(R)-3-pyrrolidinyl]oxime derivative (3-R-POD), was highly effective in various tumor cell lines in vitro, and exhibited significant tumor growth inhibition in prostate and lung xenografts in vivo. In the present study we have addressed the molecular mechanisms implicated in the anti-cancer actions of 3-R-POD. We report here that 3-R-POD induces strong apoptotic responses in A549 lung- and in DU145 prostate- cancer cells. These effects are accompanied by significant upregulation of caspase-3 activity. Focussing on A549 cells, we further demonstrate late downregulation of BCL-2 and upregulation of c-Fos gene transcription. In addition, the steroidal Na\(^+\)/K\(^+\) ATPase inhibitor induced late de-phosphorylation of Focal Adhesion Kinase (FAK) and activation of p38 MAPK. Our findings suggest that the steroidal Na\(^+\)/K\(^+\) ATPase inhibitor 3-R-POD induces apoptosis, paralleled by altered BCL-2 and c-Fos gene transcription, inhibition of the pro-survival FAK signalling, up-regulation of the pro-apoptotic p38 MAPK pathway and stimulation of caspase-3 activity.

Keywords: Apoptosis, lung cancer, prostate cancer, signaling, steroidal Na\(^+\)/K\(^+\) ATPase inhibitor.

1. INTRODUCTION

The sodium potassium pump (Na\(^+\)/K\(^+\) ATPase) is a transmembrane protein composed of multiple isoforms of catalytic α, regulatory β and modulatory γ subunits. The ion transport function of Na\(^+\)/K\(^+\) ATPase is mainly regulating ionic and osmotic balance in cells (reviewed in [1]). Recent experimental evidence points to a multi-functional role of Na\(^+\)/K\(^+\) ATPase in various cellular systems. Interestingly, this enzyme has been linked as well to cancer therapy. Indeed, recent studies established aberrant expression of some of its subunits in various malignancies including prostate, lung, colorectal, renal and pancreatic cancer [2-8]. In particular, the alpha 1 subunit of the Na\(^+\)/K\(^+\) ATPase has been reported to represent a novel target to combat glioblastoma, melanoma and non-small cell lung cancers [4-6, 8].

In a recent study, we have tested for the first time the anti-cancer activity of 17 steroidal Na\(^+\)/K\(^+\) ATPase inhibitors previously characterized as novel cardiotherapeutic agents [9]. Our results showed strong anti-cancer potencies of most compounds in vitro and nominated the 3-[(R)-3-pyrrolidinyl]oxime derivative (3-R-POD) as the most active agent in 12 different cancer cell lines; its activity peaked at GI \(50/IC\)50 and LC \(50\) values of about 10 and 100 nM respectively in proliferation assays. More importantly, the compound as a novel anti-cancer agent.

In this study, we sought to further analyze the anti-cancer properties of the 3-R-POD derivative in prostate and lung cancer cells. We have specifically focused on a series of key regulators of proliferation and apoptosis such as caspase 3, BCL-2, c-fos, FAK and p38 MAPK as these are typically involved in cancer development and progression in multiple cell systems. Our results provide novel mechanistic insights induced by 3-R-POD-dependent Na\(^+\)/K\(^+\) ATPase inhibition and collectively support further development of the compound as a novel anti-cancer agent.

2. MATERIALS AND METHODS

2.1. Chemical Synthesis and Cell Cultures

Compound 3-R-POD was synthesized in seven steps from DHEA as previously described [9]. DU145 prostate- and A549 non small cell lung- cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) or the National Cancer Institute (NCI), NIH (Bethesda, MD, USA) and were adapted to grow in RPMI 1640 (Gibco, USA) supplemented with 10% foetal bovine serum (PAA, Austria) and 1% of penicillin/streptomycin (PAA, Austria) in a 5% CO\(_2\) humidified atmosphere at 37°C. For each experiment, cells were seeded at 2 x 10\(^4\) in 6 well plates and cultured overnight. After 2 x washing with PBS, 3-R-POD (500 nM) or DMSO in fresh medium was applied for the indicated time.

2.2. FACS analysis and Caspase 3 activity

To determine the apoptotic response we used the Annexin V Apoptosis Detection Kit (MaTag, Germany). For that cells were harvested from the 6 well plate by treatment with trypsin-EDTA (Sigma-Aldrich, USA) for 10 minutes and washed once with cell culture medium. After a further wash with PBS and centrifugation at 1600 RPM for 3 minutes at RT cells were suspended in 100µl binding buffer containing Annexin V-FITC and propidium iodide and incubated for 20 minutes in the dark at RT. Subsequently, the cells were washed once, resuspended in 200µl binding buffer.
and measured immediately using the BD FACS Calibur (BD Biosciences, USA).

Active caspase-3 was ascertained by CaspGlow Fluorescein Active Caspase-3 Staining Kit (BioVision, USA) according to the manufacturers instructions. Briefly, cells detached and washed once with cell culture medium were suspended in 300µl of complete DMEM (Gibco, USA) including 1µl of FITC conjugated inhibitor of active Caspase-3 (FITC-DEVD-FMK). After 1h incubation and two washes with supplied wash buffer, cells were resuspended in 300µl of wash buffer and analyzed by flow cytometry (BD FACS Calibur, BD sciences, USA).

2.3. Western Blotting

Protein expression levels were analyzed by Western blotting. Subsequent to incubation with 500nM 3-R-POD for the indicated time periods, A549 cells were washed twice with ice-cold PBS and suspended in ice-cold RIPA lysis buffer (Thermo Fisher Scientific, USA) containing PhosSTOP phosphatase and Complete protease inhibitors (Roche Diagnostics, Germany). The extracts were centrifuged at 15000 RPM for 15 min at 4 °C and the protein concentration of the supernatant was determined by the Bradford assay (BioRad, USA). 40 µg of total proteins were boiled with Roti-Load sample buffer (Carl Roth, Germany) for 5 min at 95°C and separated in 10% SDS-PAGE. Proteins were transferred to a PVDF-membrane (Thermo Fisher Scientific, USA) and blocked for 1h at room temperature with 5% BSA (Carl Roth, Germany) in TBST. For immunostaining membranes were incubated overnight at 4 °C with antibodies directed against phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-FAK (Tyr397) and FAK (all received from Cell Signaling, USA) each diluted 1:1000 in 5% BSA. GAPDH (1:3000, Cell Signaling, USA) was used for a loading control. To detect the specific proteins membranes were incubated for 1h at RT with a 1:2000 dilution of anti-rabbit IgG conjugated to horseradish peroxidase (Cell Signaling, USA). After washing, bands were visualized using the ECL western blotting detection reagent (GE Healthcare, USA) and quantified by Quantity One Software (ChemiDoc XRS, Bio-Rad, USA).

2.4. Real-time PCR

Gene expression of BCL-2 and c-Fos was determined by real-time PCR. Total RNA was extracted from A549 cells with TriFast (Peqlab, Erlangen, Germany) according to the manufacturer’s

Fig. (1). Early and late apoptosis of A549 lung cancer cells treated by 3-R-POD. A. Chemical structure of the steroidal Na⁺/K⁺ ATPase inhibitor 3-[(R)-3-pyrrolidinyl]oxime derivative (3-R-POD). B. Flow cytometry results after a 24h treatment in the absence (control) or presence of 500nM 3-R-POD or 1µl solvent (DMSO) following staining with FITC conjugated Annexin V and propidium iodide (PI). Presented are arithmetic means ± SEM (n=6) of the percentage gated A549 cells binding: on the left bar graph Annexin V but no propidium iodide (early apoptosis), on the right bar graph both Annexin V and propidium iodide (late apoptosis). *** (p<0.001) indicates a significant difference to respective value of untreated control (unpaired t-test). C. Original dot-plots (PI/Annexin V) of a representative experiment demonstrating an increase of cell events in the lower right quadrant (early apoptosis) and upper right quadrant (late apoptosis) in 3-R-POD treated group.
Reverse transcription of 2.5 µg total RNA was performed using GoScript™ Reverse Transcription System (Promega, USA). Polymerase chain reaction (PCR) of the respective genes was set up in a total volume of 20 µl using 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix SYBR Green (Promega, USA) according to the manufacturer’s instructions. Cycling conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec, 59 °C for 15 sec and 72 °C for 30 sec. For the amplification the following primers was used (5’→3’ orientation):

- BCL-2 fw: TGGATGACTGAGTACCTGAACCG
- BCL-2 rev: TGAGCAGAGTCTTCAGAGACAGC
- FOS fw: AGACTCCTTCTCCAGCATGGG
- FOS rev: TCCTGTCATGGTCTTCACAACG
- GAPDH fw: TGAGTACGTCGTGGAGTCCACTG
- GAPDH rev: GGTGCTAAGCAGTTGGTGGTG

Analysis of a melting curve confirmed specificity of PCR products, while real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad, USA). All experiments were done in duplicate. As described earlier [10], relative quantification of gene expression was achieved using the Δct method. The amount of sample RNA was standardized by amplification of the housekeeping gene GAPDH.

2.5. Statistical Analysis

All results are presented as means ± SEM, while $n$ represents the number of independent experiments. Significance was assessed using unpaired student’s t-test or ANOVA as appropriate. $p$-values < 0.05 were considered statistically significant.

3. RESULTS

3.1. 3-R-POD Induces Strong Pro-apoptotic Responses and Caspase 3 Activation in A549 and DU145 Cancer Cells

In a previous study, using the SRB- and MTT-cell growth assays the steroidal Na⁺/K⁺ ATPase inhibitor 3-R-POD ((E, Z) 3-(((R)-pyrrolidin-3-yloxyimino)-6-methylene-5α-hydroxy-androstane-17-one, (chemical structure shown in Fig. 1A) showed potent anticancer activity in a large number of tumor cell lines [9]. These effects were attributed to its potent inhibition on the Na⁺/K⁺ ATPase (IC₅₀: 53 nM) [9]. In the present study, we addressed the pro-apoptotic effects of the compound in A549 lung- and DU145 prostate-cancer cell lines by using FACS analysis. As shown in Fig. 1B, C, strong early and late apoptosis of A549 cells became

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**Fig. (2). Early and late apoptosis of DU145 prostate cancer cells treated by 3-R-POD.** A. Flow cytometry results after a 24h treatment in the absence (control) or presence of 500nM 3-R-POD or 1µl solvent (DMSO) following staining with FITC conjugated Annexin V and propidium iodide (PI). Presented are arithmetic means ± SEM ($n$=6) of the percentage gated DU145 cells binding on the left bar graph Annexin V but no propidium iodide (early apoptosis), on the right bar graph both Annexin V and propidium iodide (late apoptosis). *** ($p<0.001$) indicates a significant difference to respective value of untreated control (unpaired t-test). B. Original dot-plots (PI/Annexin V) of a representative experiment demonstrating an increase of cell events in the lower right quadrant (early apoptosis) and upper right quadrant (late apoptosis) in 3-R-POD treated group.
evident upon treatment with 500 nM 3-R-POD for 24h. Very similar pro-apoptotic responses were also observed in DU145 cells treated with 500 nM 3-R-POD (Fig. 2A, B). In line with these findings, a significant activation of caspase 3 was observed in both cell treated for 24h with 500 nM 3-R-POD (Fig. 3A, B). These in vitro data fully support the notion that the strong anti cancer activity of this compound reported in prostate- and non-small cell lung-cancer animal efficacy studies [9] correlates with the potent pro-apoptotic responses demonstrated in both cell lines.

3.2. 3-R-POD Regulates BCL-2- and c-Fos- Gene Transcription in A549 Cancer Cells

To elucidate molecular targets that may be implicated in 3-R-POD-governed anti-cancer activity, we further tested whether this compound alters gene transcription of BCL-2, a member of the BCL-2 family of proteins that regulates anti-apoptotic responses [11]. As shown in Fig. 4A, RT-PCR analysis revealed a significant down-regulation of BCL-2 gene transcripts in A549 NSCLC cells treated with 500nM 3-R-POD. This effect was evident after a treatment of 8 hours, while in earlier time points gene transcription was not modified. We further addressed whether 3-R-POD may regulate c-Fos gene expression. In contrast to the classic concept that c-Fos is acting as an oncogene, several reports indicated that c-Fos expression may foster pro-apoptotic responses in various tumors, including ovarian and hepatocellular carcinomas [12-14]. Interestingly, RT-PCR analysis revealed that treatment of A549 cells with 500 nM 3-R-POD induced a dramatic increase in c-Fos gene transcription after 2 to 8 hours (Fig. 4B).

3.3. 3-R-POD Activates p38 MAPK in A549 Cancer Cells

p38 MAPK is a mediator of apoptosis in response to a number of cellular signals (for a recent review see [15]). Various transcriptional and posttranscriptional mechanisms, which can involve the regulation of apoptotic and/or survival pathways, control the p38 MAPK-initiated pro-apoptotic responses. Accordingly, we further addressed the involvement of p38 MAPK in 3-R-POD-induced apoptosis in A549 lung cancer cells. As shown in Fig. 5A, a significant increase in p38 MAPK phosphorylation became evident after long term (8 hours) treatment of A549 cells with 3-R-POD.
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**Fig. 5.** Time dependent effects of 3-R-POD on p38 MAPK activity in A549 lung cancer cells. A549 cells were stimulated with 500 nM 3-R-POD for the indicated time periods. Following cell lysis, equal amounts of total lysates were immunoblotted with a specific antibody. Protein abundance was analyzed by densitometry. A. Intensity of phospho-p38 MAPK (Thr180/Tyr182), normalized to the intensity of the corresponding total p38 MAPK band. Bars indicate the relative fold increases as arithmetic means ± SEM from n=4 independent experiments. ** indicates p<0.01 to respective value of untreated control (unpaired t-test). Below, representative original western blots showing the protein abundance of phosphorylated (p, Thr180/Tyr182) p38 MAPK, total MAPK and respective GAPDH as loading control. B. Intensity of phospho-FAK (Tyr397) normalized to the intensity of the corresponding total FAK band. Bars indicate the relative fold increases as arithmetic means ± SEM from n=6 independent experiments. * indicates p<0.05, *** indicates p<0.001 to respective value of untreated control (unpaired t-test). Below representative original western blots showing the protein abundance of phosphorylated (p,Tyr397) FAK, total FAK and respective GAPDH as loading control.

3.4. 3-R-POD Down-regulates FAK Phosphorylation in A549 Cancer Cells

We further analyzed the capacity of 3-R-POD to regulate the activity of key signaling kinases implicated in cell survival and growth. For this we addressed the phosphorylation state of the Focal Adhesion Kinase FAK. As previously described [16], FAK regulates maintenance of normal cell survival, while disruption of FAK signaling controls various cellular outcomes including loss of substrate adhesion and apoptotic responses [16]. As shown in Fig. 5B, the ratio of phosphorylated FAK to the total protein content dropped significantly after long term (8 hours) incubation of the cells with 500 nM 3-R-POD, indicating inactivation of this key signaling kinase.

4. DISCUSSION

Na⁺/K⁺ ATPase is an emerging target in anti-cancer therapy, currently exploited in the development of novel therapeutics (reviewed in [2-6, 8]). Analysis of the molecular mechanisms mediating the anti-cancer properties of known Na⁺/K⁺ ATPase inhibitors (e.g. sugar-containing, plant-derived inhibitors such as digoxin), defined effects of these inhibitors in specific proliferation, survival, metabolism, angiogenesis and cell attachment pathways in cancer cells; these included effects on multiple molecular targets such as Myc, NF-κB, HIF-1, p53 and others (reviewed in [2-6, 8]).

Previous studies in our laboratory have focused on the characterization of the anti-cancer activity of a series of non-sugar containing, steroidal Na⁺/K⁺ ATPase inhibitors originally developed as novel cardiotherapeutic agents [17-21]. These studies reasonably correlated the potency of the observed Na⁺/K⁺ ATPase inhibition with anti-cancer activity in multiple cells [9]. Specifically, it was shown that the presence of a methylene or a methoxylimino functionality at C6 in combination with a (R)-pyrrolidin-3-oxime derivative (3-R-POD, Fig. 1A) was identified as a potent Na⁺/K⁺ ATPase inhibitor with strong anti-cancer action in prostate and lung cancer cells and their corresponding xenografts in vivo [9]. In the current study, we present novel mechanistic insights underlying the potent anti-cancer activity reported for 3-R-POD. Specifically, we show that treatment of DU145 prostate- and A549 NSCLC- tumor cell lines with 500nM 3-R-POD induced: a) strong pro-apoptotic responses and b) significant activation of caspase 3. Furthermore and in line with these findings, the steroidal inhibitor was shown to: c) down-regulate BCL-2- and up-regulate c-Fos gene transcription and d) inhibit FAK- and trigger p38 MAPK-activity in A549 NSCLC- cells. Although additional studies will be required to determine whether the reported effects on signalling pathways are directly linked to 3-R-POD-dependent Na⁺/K⁺ ATPase inhibition, our findings clearly expand the current knowledge in the field by linking, for the first time, several gene products and proteins involved in cell survival and apoptosis with Na⁺/K⁺ ATPase inhibition.

Apoptotic cell death is regulated by complex interactions between pro-survival and pro-apoptotic members of the B-cell lymphoma-2 (BCL-2) protein family [22]. BCL-2 apoptosis-related family members are involved in the regulation of cellular fate as a response to antineoplastic agents and treatments in tumors (for recent reviews see [23, 24]). BCL-2 family proteins have been considered as drug targets in tumor biology [25], while BCL-2 antagonists have been recently proposed for cancer therapy [26]. The downregulation of BCL-2 gene transcription upon treatment of
A549 NSCLC cells by 3-R-POD is in line with these reports and support the notion that the steroidal Na+/K+ ATPase inhibitor may be a potent agent implicated in BCL-2 apoptosis-related induction of cell death.

Although the classic concept for c-Fos activity is based on its oncogenic properties, several studies in recent years indicate that c-Fos may also have tumor-suppressor activity. Indeed, c-Fos was shown to act as a proapoptotic agent in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in prostate cancer cells [27]. In addition, c-Fos has been proposed to act as a mediator of the c-myc-induced apoptotic signaling in hepatocellular carcinomas via the p38 mitogen-activated protein kinase pathway [13]. Moreover, it has been reported that loss of c-Fos expression was associated with tumor progression in epithelial ovarian carcinomas [14]. In another study, fenretinide (HPR) that exhibits preventive and therapeutic activity against ovarian tumors was reported to cause induction of c-Fos gene expression in ovarian carcinoma cells [12]. This study established a relationship between c-Fos induction and apoptosis by the finding of an increased apoptotic rate in cells overexpressing c-Fos [12]. Our results showing strong induction of c-Fos gene transcription in 3-R-POD-treated A549 NSCLC cells that undergo potent apoptosis fully support the postulated relationship between c-Fos induction and apoptosis. Although further studies are now required to identify the molecular link of c-Fos up-regulation with apoptotic signaling in lung cancer cells, our findings showing potent p38 MAPK activation imply potential involvement of c-Fos-regulated MAPK apoptotic signaling in 3-R-POD-induced effects.

The regulation of p38 MAPK apoptotic function is controlled by different signaling pathways [28]. Classically, p38 MAPK is activated by the small Rho family GTPases Rac1 and Cdc42 [29] and can mediate apoptosis or cell survival, by mechanisms involving the regulation of the expression and/or activity of different members of the Bcl-2 family. Indeed, Bax a pro-apoptotic member of the Bcl-2 family can be regulated by p38 MAPK. For example, in cardiomyocytes and MEFs-derived cell lines, p38 MAPK controls apoptosis, induced by different stimuli, through up-regulation of the pro-apoptotic proteins Fas and Bax and down-regulation of the activity of ERKs and Akt pro-survival pathways [30-31]. In lung cancer cells, p38 MAPK was shown to mediate isothiocyanate-induced strong pro-apoptotic response [32], while a critical role of the p38-mediated signaling pathway in the determination of lung cancer cell's sensitivity to EF24, a fluorinated synthetic curcumin analog was recently reported [33]. Our findings showing that the steroid inhibitor 3-R-POD activates p38 MAPK signaling and down-regulates of BCL-2 gene transcription in A549 lung cancer cells are in line with these reports and provide additional experimental evidence for the involvement of the p38 MAPK pathway in apoptosis regulation in response to Na+/K+ ATPase inhibition in lung cancer cells.

FAK is a nonreceptor protein-tyrosine kinase mainly localized within focal adhesions. A wide variety of extracellular signals including growth factors, steroid hormones, cytokines and neuropeptides have been reported to phosphorylate and thus activate FAK [34-37]. FAK signaling in turn is further activating downstream molecules such as PI-3K/Akt [38, 16] and paxillin [39]. Subsequent studies over the past years have established that FAK is also important in maintaining normal cell survival and cell adhesion, while inhibition of FAK activity and signaling results in loss of substrate adhesion and apoptosis (for recent reviews see [40, 38, 16]). Thus FAK may represent a potential target molecule for drug discovery in human cancers (for recent reviews see [41, 42]). Our findings showing 3-R-POD-induced inhibition of FAK phosphorylation are in line with the observed potent apoptotic responses of prostate- and lung-cancer cells upon stimulation with the steroidal Na+/K+ ATPase inhibitor 3-R-POD and further support the notion that inhibition of FAK activity and signaling comprises important anti-tumorigenic potential.

In conclusion, this study implicates for the first time a series of important regulators of cell survival and apoptosis with Na+/K+ ATPase inhibition by 3-R-POD in prostate and lung tumor cells. Moreover, our results, when taken into consideration with previous observations showing higher selectivity and therapeutic index versus digoxin and potent anti-tumor efficacy in prostate and lung xenografts [9], clearly support the favourable properties of 3-R-POD and warrant its further anti-cancer development. This is line with previous observations by Gobbini and co-workers [20] showing that a family of steroidal inhibitors comprising 3-R-POD has 5-20 times higher cardiotherapeutic (inotropic) activity and significantly less toxicity (3-10 fold) in comparison to digoxin in guinea pig studies [20]. Future studies aiming to characterize additional effects of 3-R-POD in cells and assess its potential impact on non-pump related functions of the Na+/K+ ATPase are currently in progress.

AUTHORS' CONTRIBUTIONS


CONFLICT OF INTEREST

The authors declare that they have no competing interests towards any aspect of the work described in this paper.

ACKNOWLEDGEMENTS

Authors would like to thank Tanja Loch for the careful preparation of the manuscript. We are also grateful to Dr. E.A. Papakonstanti for noteworthy comments. This work was supported by grants from the University of Crete Research Committee (KA 3452 to CS), Medexis Biotech SA (to KA), Deutsche Forschungsgemeinschaft (SFB773 to FL) and the Deanship of Scientific Research at King Saud University (Research Group Project No. RGPVPP-018 to SA, SA, and CS).

LIST OF ABBREVIATIONS

3-R-POD = 3-[1R]-3-pyrrolidinyl]oxime derivative
FAK = Focal Adhesion Kinase
Na+/K+ ATPase = Sodium potassium pump
NSCLC = Non Small Cell Lung-Cancer
BCL-2 = B-cell lymphoma-2
TRAIL = Tumor necrosis factor-related apoptosis-inducing ligand

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A Steroidal Na+/K+ ATPase Inhibitor Triggers Pro-apoptotic Signaling

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Received: April 11, 2014  Revised: June 10, 2014  Accepted: June 16, 2014