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Steroidal Cardiac Na^+/K^+ ATPase Inhibitors Exhibit Strong Anti-Cancer Potential *in vitro* and in Prostate and Lung Cancer Xenografts *in vivo*

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Abstract: Sodium potassium pump (Na^+/K^+ ATPase) is a validated pharmacological target for the treatment of congestive heart failure. Recent data with inotropic drugs such as digoxin & digitoxin (digitalis) suggest a potent anti-cancer action of these drugs and promote Na^+/K^+ ATPase as a novel therapeutic target in cancer. However, digitalis have narrow therapeutic indices, are pro-arrhythmic and are considered non-developable drugs by the pharmaceutical industry. On the contrary, a series of recently-developed steroidal inhibitors showed better pharmacological properties and clinical activities in cardiac patients. Their anti-cancer activity however, remained unknown. In this study, we synthesized seventeen steroidal cardiac inhibitors and explored for the first time their anti-cancer activity *in vitro* and *in vivo*. Our results indicate potent anti-cancer actions of steroidal cardiac inhibitors in multiple cell lines from different tumor panels including multi-drug resistant cells. Furthermore, the most potent compound identified in our studies, the 3-[(*R*)-3-pyrrolidinyl]oxime derivative **3**, showed outstanding potencies (as measured by GI_{50}, TGI and LC_{50} values) in most cells *in vitro,* was selectively cytotoxic in cancer versus normal cells showing a therapeutic index of 31.7 and exhibited significant tumor growth inhibition in prostate and lung xenografts *in vivo*. Collectively, our results suggest that previously described cardiac Na^+/K^+ ATPase inhibitors have potent anti-cancer actions and may thus constitute strong re-purposing candidates for further cancer drug development.

Keywords: Na^+/K^+ ATPase, steroidal cardiac inhibitors, multi-drug resistant cells, lung tumors, prostate tumors.

INTRODUCTION

The sodium potassium pump (Na^+/K^+ ATPase) is a trans-membrane protein complex composed of multiple isoform combinations of catalytic α, regulatory β and modulatory γ subunits acting as a key energy driver maintaining ionic and osmotic balance in cells (reviewed in [1]). Na^+/K^+ ATPase functions as a receptor for cardiotonic steroids (CTS), a family of chemical compounds comprising a steroidal nucleus substituted by a sugar moiety and a lactone group at positions 3 and 17 respectively (reviewed in [2]). CTS include more than 50 different plant-derived compounds but are typically exemplified by digoxin and digitoxin, two generic drugs currently approved as pharmacological Na^+/K^+ ATPase inhibitors for the treatment of congestive heart failure (reviewed in [2]).

Although the ion transport function of the Na^+/K^+ ATPase is well documented, recent evidence points to a multi-factorial role of the enzyme in cells. For example, Na^+/K^+ ATPase has been implicated in multiple signaling pathways acting as a transducer and/or integrator of various signals including MAPK, ROS, phospholipase C and inositol triphosphate receptor (IP3R) [3] or as a protein-protein interaction scaffold forming signal receptor complexes with proteins like src or caveolin-1 [4, 5]. Moreover, Na^+/K^+ ATPase has been implicated in diverse cellular functions including adhesion, motility and actin dynamics [6-10]. Most recently, Na^+/K^+ ATPase has been linked to cancer therapy as aberrant expression of some of its subunits has been observed in a growing number of cancers including prostate, lung, colorectal, renal, pancreatic and others [11-20]. Interestingly, up-regulation of the α1 isoform, increase in the ratio of α3 to α1 isoforms and down regulation of β1 isoforms have been reported in cancer cells, pointing to a complex regulation of Na^+/K^+ ATPase holoenzyme formation in different cells (reviewed in [21]).

A series of additional experimental and other data further validate Na^+/K^+ ATPase as an emerging target in cancer treatment. First, epidemiological data in cardiac patients treated with digoxin/ digitoxin (also known as digitalis) showed reduced breast cancer incidence and subsequent mortality in patients of the Nordic region [22]. Second, a study in 9271 patients correlated high levels of plasma digitoxin with a low risk of leukemia/lymphoma [23]. Finally, a study in 47.884 digoxin-treated men followed for 20 years showed lower prostate cancer development risk [24]. Based on these properties, several groups have obtained preclinical anti-cancer efficacy data with digoxin & other CTS attributed to effects on various proliferation, survival, metabolism, angiogenesis and cell attachment pathways in cancer cells (reviewed in [25]).

Despite their long clinical experience in cardiac indications and promising anti-cancer activities, digitalis compounds suffer from a narrow therapeutic index and an increased risk for arrhythmia induction (reviewed in [26]). Other CTS properties such as extremely long half-lives (digoxin: 30-50h, digitoxin: 5-8 days) and increased potential for drug-drug interactions [27] further limit their potential use in therapy. To overcome these problems, several groups focused on the development of second generation cardiac Na^+/K^+ ATPase inhibitors with improved therapeutic indices. A promising class of novel, non-sugar containing, steroidal compounds exemplified by clinical phase II cardiac drug istaroxime was consequently described and tested in various preclinical and clinical trials with promising results [28-32]. However, the potential anti-cancer activity of these compounds remained unknown to date

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as these compounds were exclusively developed and tested for cardiac indications.

In this study, we have synthesized, characterized and tested for the first time 17 steroidal Na^+/K^+ ATPase cardiac inhibitors [28-31] for their anti-cancer activity *in vitro*. Istaroxime was also included in all our experiments; corresponding results will be presented in a separate publication (manuscript in preparation). Based on an activity profile established using a pre-screen comprising 3 NCI (National Cancer Institute, USA) cancer cell lines, we have shown potent anti-cancer potencies of the compounds *in vitro*. Focusing on compound **3**, the most potent inhibitor identified in our studies, we have further characterized its effects in a total of 7 tumor panels comprising 12 different cancer cell lines and performed anti-cancer efficacy studies in prostate and non-small cell lung cancer animal models. Altogether our results revealed, for the first time, a strong anti-cancer potential of the tested cardiac inhibitors *in vitro* and *in vivo*. Based on these properties, tested cardiac inhibitors and compound **3**, in particular, may be strong re-purposing candidates for anti-cancer drug development.

MATERIALS AND METHODS

Cardiac compounds used in these studies have been synthesized with modifications as previously described [28-32]. Details on their synthesis and characterization are provided in the Supplementary Materials & Methods Section.

Cell Lines

All cancer 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 RPMI1640 supplemented with 25 mM HEPES, 2 mM L-Glutamine, 5-10% fetal bovine serum and antibiotics in a 5% CO_2 humidified atmosphere at 37°C. The following human cancer cell lines have been included in this study: prostate: PC-3, DU145; lung: A549, EKVX, Central Nervous System: SF-268, renal: CAKI-1, melanoma: SK-MEL28, MDA-MB-435; ovarian: OVCAR-3, OVCAR-5 and NCI-ADRES; colon: HCT-116. Normal human fibroblast cells were obtained from Lonza (USA).

Na^+/K^+ ATPase Assays

The compound inhibitory effect on ATPase activity was assessed *in vitro* using the Adenosine 5'-Triphosphatase Enzymatic Assay of Sigma (St.Louis, MO) according to the manufacturer's instructions. This assay utilizes enzyme isolated from porcine cerebral cortex. Each reaction was performed in a final volume of 250 µl using 0.5 units/ml enzyme in the presence of inhibitory compounds. For the determination of IC_{50} values, each inhibitor was added in five concentrations ranging from 10^{-5} to 10^{-9}M (in triplicates). IC_{50} values were calculated using the Origin® software (OriginLab, Northampton, MA).

SRB Assays & Development Therapeutics Program Graph Analysis

Sulforhodamine B (SRB) assays were performed according to NCI guidelines for anti-cancer drug screening (http://dtp.nci.nih.gov/branches/btb/ivclsp.html) and as previously published [33]. A detailed SRB protocol is included in the Supplementary Materials & Methods Section. Analysis of SRB results to generate Mean Graphs was done using the publicly available function of the COMPARE tool program of the NCI, Development Therapeutic Program branch (http://dtp.nci.nih.gov/compare-web-public_compare/SearchAndDisplay/SearchAndDisplay.do). This tool allows plotting the SRB-assay calculated GI_{50} and/or LC_{50} values into graphs where activity values are expressed in a log scale; negative values are indicative of cell inhibition and/or elimination. Increasingly negative values denote highly active compounds (according to the "forward direction" data analysis tool available at the COMPARE site). Average activities for different compounds in log scale ("MG-MID") can thus be generated as based on individual cell line log values.

MTT Assays

Cell proliferation/viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays (Sigma, St.Louis, MO). Cells were cultured in 96-well plates for 24h (7-10.000 cells/well) and were subsequently incubated with 5 concentrations (in triplicates) of each drug (ranging from 10 nM–100 µM) or DMSO control in serum-containing medium for 48-72h. At the end of the incubation, medium was aspired and MTT dissolved in RPMI1640 was added to a final concentration of 0.25 mg/ml. After 4h incubation (37°C, 5% CO_2, dark) the supernatant was discarded, the converted dye (blue formazan crystals) was solubilized by adding 200 µl DMSO and absorbance was measured at 550 nm with reference at 655 nm using a spectrophotometer. IC_{50} compound values were calculated using the Origin® program software (OriginLab, Northampton, MA). Note that MTT-calculated IC_{50} values may differ from SRB-assay calculated GI_{50} values due to inherent differences in assay methodologies.

Maximum Tolerated Dose (MTD) Assays

Female mice, 8–9 weeks old, weighing 20-21 g were used for the MTD studies according to NCI guidelines; these are based on use of a single animal per dose in an effort to minimize the number of animals used in experiments and conserve compound (http://dtp.nci.nih.gov/branches/btb/acute_tox.html). We have thus injected one animal per individual dose with 50/25/12.5 mg/kg of compound **3** respectively, in a volume of 20µL/g of weight. Administrations were done intraperitoneally once daily at a time interval of 24h for consecutive injections. The administrations were as follows:

50 mg/kg: 1 injection (schedule: Q1D1, i.e. one injection per day for 1 day); 25 and 12.5 mg/kg: 3 injections (schedule Q1D3, i.e. one injection per day for 3 days).

Animals were weighed prior to each administration and volumes/dose administered were adjusted according to body weights. The animal that received 50 mg/kg suffered from sedation that got progressively aggravated within 48h; the animal was thus sacrificed. No side effects were recorded for the remaining two animals that received 25 and 12.5 mg/kg; these animals subsequently received two additional injections at 24h time intervals. Both animals remained healthy without any obvious changes in behavior/motility/reactions/weight after the three doses and up to one week after the first injection. Based on these studies, we concluded that the compound's MTD was approximately 25 mg/kg.

Pharmacokinetic Experiments

All pharmacokinetic experiments were performed by Medicilon Contract Laboratory (US/CN). Briefly, 2 groups of 24 male BALB/c-nude mice were injected with 10 or 25 mg/kg of compound **3** *via* intravenous (bolus injection *via* the lateral tail vein) or intra-peritoneal routes respectively. Blood samples (~300 µL/ sample) were collected *via* cardiac puncture after euthanasia by carbon dioxide inhalation in tubes containing K2-EDTA and were stored on ice until centrifuged at 8000 rpm for 6 minutes at 2-8°C. The resulting plasma was separated and stored frozen at -80°C prior to analysis. Overall, 8 time points were chosen: at post-dose at 5 min, 15 min, 30 min, 1 h, 2h, 4 h, 7h and 24h. Each time point comprised pooled samples from 3 mice. Isolated plasma samples were analyzed by standard LC-MS/MS. The analytical results were confirmed using quality control samples for intra-assay variation (within day variation).

A standard set of parameters including Area Under the Curve ($AUC_{(0-t)}$, $AUC_{(0-\infty)}$), elimination half-live ($T_{1/2}$), maximum plasma concentration (C_{max}), time to reach maximum plasma concentration (T_{max}), clearance (CL), and volume of distribution (V_z) were calculated using non-compartmental analysis modules in FDA-

certified pharmacokinetic program WinNonlin Professional v5.2 (Pharsight, USA). Furthermore, the bioavailability was estimated using the following formula:

$$F = \frac{AUC_{D0\text{-}\infty E(PO)} \times Dose_{IV}}{AUC_{D0\text{-}\infty E(IV)} \times Dose_{(IP)}} \times 100\%$$

Where:

$AUC_{(0\text{-}t)}$	Area under the curve from the time of dosing to the last measurable concentration
$AUC_{(0\text{-}\infty)}$	Area under the curve from the time of dosing extrapolated to infinity, based on the last observed concentration
CL	Total body clearance, CL=Dose/AUC
C_{max}	Maximum observed concentration, occurring at T_{max}
F	Bioavailability
$MRT_{(0\text{-}\infty)}$	Mean residence time from the time of dosing to infinity
T_{max}	Time of maximum observed concentration
$T_{1/2}$	Terminal half-life = ln(2)/λz
V_z	Volume of distribution based on the terminal phase

Xenograft Studies

Xenografts were generated by subcutaneously injecting exponentially growing cultures of ~10^6 PC-3 or A549 cells at the axillary region of 6-8 weeks old male (PC-3) or female (A549) Nod/Scid mice according to the British practice of bilateral trocar implants which offers reduced variability and data accuracy based on small numbers of animals [34]. Following development of palpable tumors and group randomization, all compounds were injected intraperitoneally (see details below). Tumor volumes were calculated according to the formula [(axb2)/2], where a=length and b=width of the tumor as measured with a vernier's caliper (measurements performed twice a week). %DT/DC values were also calculated, where DT = T − Do and DC = C − Do (Do is the average tumor volume at the beginning of the treatment; T and C are the volumes of treated and untreated tumors, respectively, at a specified day). DT/DC values below 42% represent highly active compounds according to NCI guidelines. Losses of weight, neurological disorders, behavioral and dietary changes were also recorded as indicators of toxicity (side effects). Experiments were terminated when tumor sizes in untreated animals reached a volume of ~1000-1500 mm^3. Paclitaxel, a mitotic inhibitor approved in the clinic -among other indications- for lung and prostate cancer was included in both xenograft studies as a clinically-relevant positive control [35].

PC-3 Experiments

When the mean tumor volume reached an average of 176.7 mm^3 at day post inoculation (dpi) 13 in all animals, mice were randomly divided into 4 groups of 6 animals:

Group A: untreated animals.

Group B: paclitaxel 20 mg/kg (Cremophor/EtOH/WFI: injected at dpi 13/17/21/24/28).

Groups C and D: 25 mg/kg of Compound **3** (WFI; injected at dpi 13-15/20-22/27-29) and 17 mg/kg of Compound **3** (WFI; injected once daily, dpi 13-30), respectively.

Group E: 2 mg/kg digoxin (PBS); injected once daily, dpi 13-30.

A549 Experiments

When the mean tumor volume reached an average of 117.6 mm^3 (dpi 20) in all animals, mice were randomly divided into groups of 6 (vehicle) or 7 animals (treatment groups):

Group A: untreated animals.

Group B: paclitaxel 18 mg/kg (Cremophor/EtOH/WFI; injected at dpi 21/24/28/31/35/38/42/45/49/52).

Group C, D and E: 20 mg/kg of Compound **3** (WFI; injected at dpi 20/21-24/27-28/34-35/38/41-42/45/48-52) 15 mg/kg of Compound **3** (WFI; dpi 20-24/27-29/34-38/41-45/48-52) and 10 mg/kg of Compound **3** (WFI; injected once daily for 5 consecutive days followed by 2 days rest for a total of 5 weeks starting at dpi20 [scheme (Q1Dx5;2) x5]), respectively).

WFI: water for injection

Ethical Conduct of Animal Experiments

MTD and xenografts experiments were performed at the Pharmacy Department, University of Athens, Greece under the approval of the veterinary committee (Approval number K/2844) and in agreement with Greek laws (2015/92), EU & European council guidelines (86/609 and ETS123, respectively), and Compliance with Standards for Human Care and Use of Laboratory Animals, NIH, USA (Assurance No. A5736-01).

Animals used in pharmacokinetic studies were treated according to the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Academy Press, Washington, 1996. Specific conditions regarding handling of moribund animals as determined by the veterinary staff of the test facility were explicitly defined in the study protocol according to international guidelines including euthanasia for humane reasons. This included carbon dioxide inhalation followed by exsanguination. Final disposition of all animals placed on study was documented in all study records.

RESULTS

Compound Structures and Na^+/K^+ ATPase IC_{50} Inhibitory Activities

The structures of all tested compounds are depicted in Supplementary Table **1**. Overall, these include several oximes at the position C3 (R^1) of the carbon skeleton in combination with different substitutions at carbons C5, C6, C7 and C17 (R^2, R^3, R^4 and R^5 respectively) [28-31]. Screening of IC_{50} inhibitory activities of all compounds, as determined by *in vitro* Na^+/K^+ ATPase assays using purified porcine brain enzymes, are presented in Supplementary Table **2** ("NaK Assay" column). With the exception of compound **14** which had an IC_{50} activity superior to 10 μM, all other tested inhibitors had IC_{50} activities below 1 μM. Some of the inhibitors were extremely potent showing activities below 0.1 μM (e.g. compounds **2**, **3**, **10** and **17**). In comparison, digoxin had an IC_{50} of 0.2 μM in the same assay. These results were roughly comparable to IC_{50} measurements performed previously with the same compounds using purified dog ATPase [28-31].

In Vitro Anti-Cancer Activity of Na^+/K^+ATPase Inhibitors

Having established the Na^+/K^+ ATPase inhibitory activity of compounds 1-17, we set to characterize their *in vitro* anti-cancer potency in a pre-screen comprising 3 cancer cell lines in agreement with NCI guidelines for testing of anti-cancer agents. Specifically, we determined GI_{50}, TGI and LC_{50} parameters (concentrations to achieve respectively Growth inhibition of 50%, Total Growth Inhibition and Lethal Concentration to eliminate 50% of the cells, see Materials and Methods) of the compounds in non-small cell lung (A549), prostate (PC-3) and central nervous system (SF-268) cancer cell lines. Digoxin was added as a control. The results (Supplementary Table **2**, "SRB assay" columns) showed high potency of most of the compounds. We have also independently validated the SRB results in MTT cell growth assays in 2 additional cancer cell lines shown to express Na+/K+ATPase, namely prostate (DU145) and renal (CAKI-1) cells [36]. Similar to SRB results, MTT assays revealed high activity of most of the compounds

(Supplementary Table **2**, "MTT assay" columns). Based on these assays, compound **3** (Fig. **1**, panel A) was deemed the most potent agent within this series of 17 cardiac Na^+/K^+ ATPase inhibitors, showing GI_{50} and LC_{50} values below 1 nM and 100 nM in SRB assays respectively and IC_{50} values below 0.01 μM in MTT assays (Supplementary Table **2**).

In Vitro Anti-Cancer Action of Compound 3 in Multiple Cell Lines

Focusing on compound **3**, the most potent inhibitor identified in the pre-screening analysis, we further characterized the anti-cancer action of this compound and compared it with that of digoxin. SRB assays in 9 cell lines derived from 6 tumor panels (lung, melanoma, ovarian, prostate, colon and renal) were used for this analysis. A human non-cancer fibroblast cell line was included as a cytotoxic cell control. As shown in Fig. (**1**) and Supplementary Table **3**, compound **3** was highly active in all cell lines tested including NCI-ADRES cells, a cellular model of multi-drug resistance due to overexpression of high levels of MDR1 and P-glycoprotein [37, 38]. In comparison to digoxin, compound **3** exhibited superior GI_{50}, TGI and LC_{50} values in all cell lines. As shown in Fig. (**1**), panels B, C, graphically plotting activity of both compounds according to NCI's Development Therapeutics Program COMPARE tools that depict absolute compound potency in a log scale for better visualization (See Materials and Methods) revealed an average difference of more than one log in cytotoxic activity (LC_{50}) in favor of compound 3 in the 12 cell lines tested (-6.82 ± 0.937 versus -5.74 ± 0.973 of digoxin respectively, Fig. (**1**) panels B and C; negative values denote increasing tumor cell elimination). More importantly, compound **3** was selectively cytotoxic in cancer cells versus normal fibroblasts as the corresponding average LC_{50} values in all cancer cell lines tested were 31.7 times lower than the LC_{50} value recorded in normal fibroblasts (Fig. **1**, panel D). In sharp contrast, digoxin was more cytotoxic in normal cells than in cancer cells confirming the narrow therapeutic index of this compound (Fig. **1**, panel D). Similar results were obtained in HEK293 fibroblasts

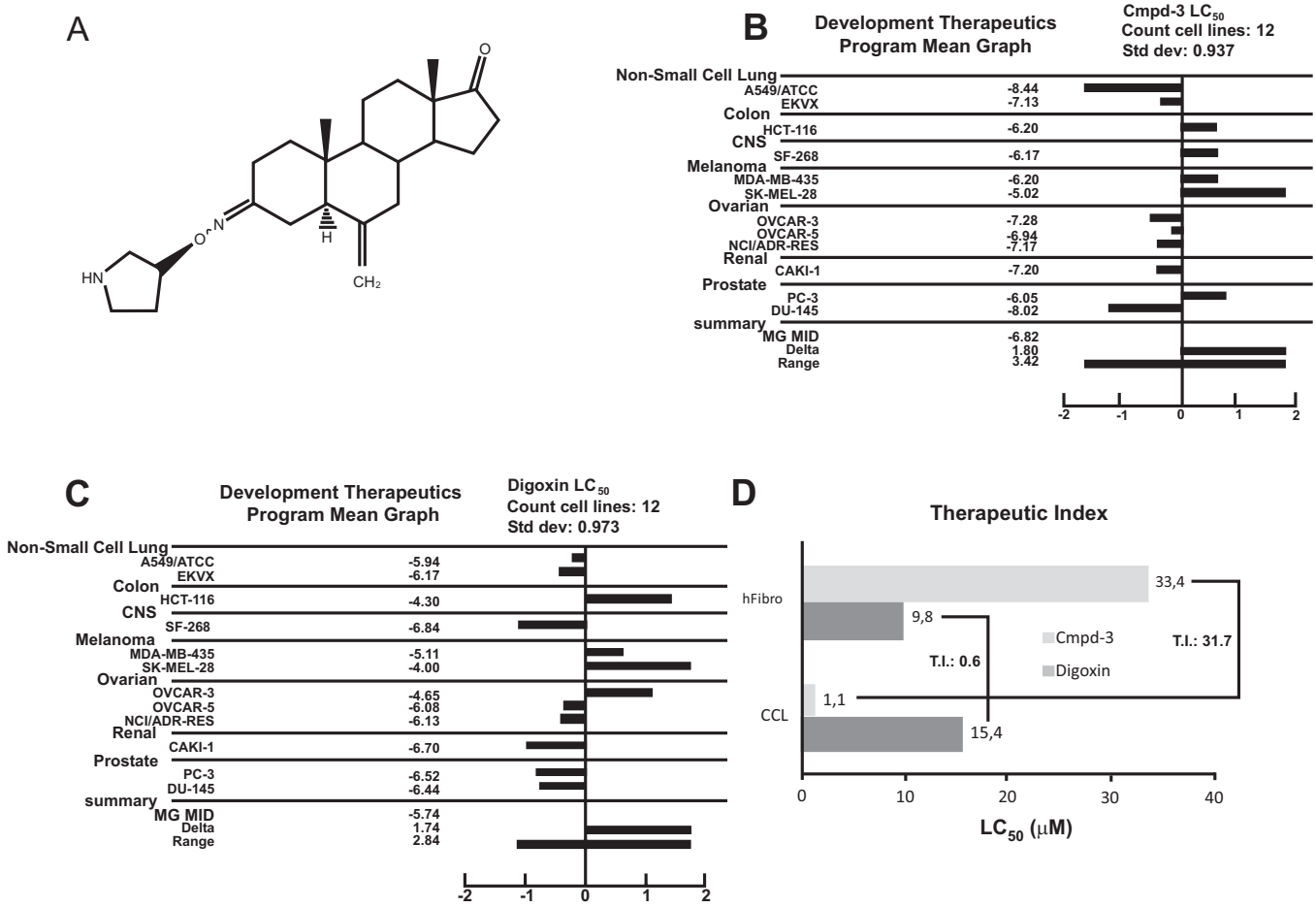

Fig. (1). Panel A: Chemical structure of (E, Z) 3-((R)-pyrrolidin-3-yloxyimino)-6-methylene-5α-androstane-17-one (Compound **3**). Panels B and C: LC_{50} activity plots generated using the NCI's Development Therapeutics Program COMPARE tool. The graph depicts, in log scale, compound activities in 12 cancer cell lines as based on LC_{50} values of Supplementary Tables **2** and **3** (i.e. average LC_{50} values from 2-3 independent SRB experiments, see Supplementary Tables **2** and **3**); MG-MID: average LC_{50} values calculated for each compound as based on the individual activity values in log scale in each cell line (MG-MID for compound **3**: -6.82; MG-MID for digoxin: -5.74); Std dev: standard deviation for the aforementioned values (compound **3**: 0.937, digoxin: 0.973); Delta: denotes the highest difference observed in a cell line in comparison to the calculated mean value in all cell lines; Range: denotes the difference in log scale between the highest and lowest activity observed. Overall, higher negative values denote increasing capacity of tumor cell elimination (see Materials and Methods). Panel D: Therapeutic index calculation of compounds. Calculations are based on average LC_{50} values in μM observed in SRB assays of compound **3** and digoxin in 12 cancer cell lines (CCL) versus the LC_{50} values in μM observed with the compounds in human fibroblasts (hFibro). Compound **3** had an average LC_{50} of 1.1 μM in CCL versus 33.4 μM in hFibro (resulting in a therapeutic index of 31.7). On the contrary, digoxin had an average LC_{50} of 15.4 μM in CCL versus 9.8 μM in hFibro (resulting in a therapeutic index of 0.6). Overall therapeutic index values<1 denote non-selective compound cytotoxicity, whereas values>10 denote selective action in cancer cells. Of note, therapeutic index values, unlike log values presented in Panels B and C, are positive as these are calculated based on a simple division of LC_{50} values in μM of each compound.

(data not shown). Taken together our results suggest that compound **3** has a strong anti-cancer action in multiple cell lines including multi-drug resistant cells.

Compound 3 Shows Strong Anti-Cancer Action in Animal Xenografts

To further characterize the anti-cancer properties of compound **3**, we selected prostate PC-3 and lung A549 cancer cells to generate xenograft models *in vivo*; these cells were highly receptive to the compound's actions, as mentioned above. Initially, we determined the maximum tolerated dose (MTD) of the compound by injecting increasing doses in NOD/SCID mice *via* intraperitoneal (IP) injection; these studies established reasonable safety margins for a dose of 25 mg/kg (see Materials and Methods). Subsequent pharmacokinetic experiments using the defined MTD dose of 25 mg/kg injected intraperitoneally in male Balb/c-nu mice, established high availability of the compound in plasma reaching a C_{Max} of 5014.8 µg/L with a half-life of 7.52h. When the compound was injected *via* the intravenous route at a dose of 10 mg/kg, a C_{Max} of 2737.83 µg/L and a half-life of 4.89h was calculated (Table **1**). Overall, compound's **3** high plasma bioavailability and relative rapid elimination (half-lives lower than 8h) as observed in our experiments, are compatible with a lower potential of toxicity and drug drug interactions *in vivo* unlike digoxin [27]. Choosing the IP route for animal experiments due to repetitive dosing, we showed that in xenografts generated by subcutaneous injection of PC-3 cells in male mice, compound **3** had a strong, dose dependent anti-cancer activity with DT/DC values continuously lower than the NCI activity cut-off of 42% (Fig. **2**; Panels A, C). The activity of compound **3** at the highest dose was comparable to paclitaxel, a

Table 1. Selected Pharmacokinetic parameters of compound 3 in mice following intravenous and intraperitoneal administration.

	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	$MRT_{(0-\infty)}$	$t_{1/2}$	T_{max}	Vz	CL	C_{max}	F*
	µg/L*h	µg/L*h	h	h	h	L/kg	L/h/kg	µg/L	%
Compound 3									
IV-10 mg/kg	12271.44	12625.59	6.00	4.89	0.083	5.10	0.72	2737.83	
IP-25 mg/kg	29151.10	32170.67	9.00	7.52	0.083	NA	NA	5014.80	101.92

AUC (0-t): Area under the curve from the time of dosing to the last measurable concentration; AUC (0-∞): Area under the curve from the time of dosing extrapolated to infinity, based on the last observed concentration; CL: Total body clearance (CL=Dose/AUC); Cmax: Maximum observed concentration, occurring at Tmax; F: Bioavailability; MRT (0-∞): Mean residence time from the time of dosing to infinity; Tmax: Time of maximum observed concentration; $T_{1/2}$: Terminal half-life = ln(2)/λz; Vz: Volume of distribution based on the terminal phase. Note that the F value is missing in the IV line since the levels achieved after IV administration are used as a reference to calculate the F value after IP administration.

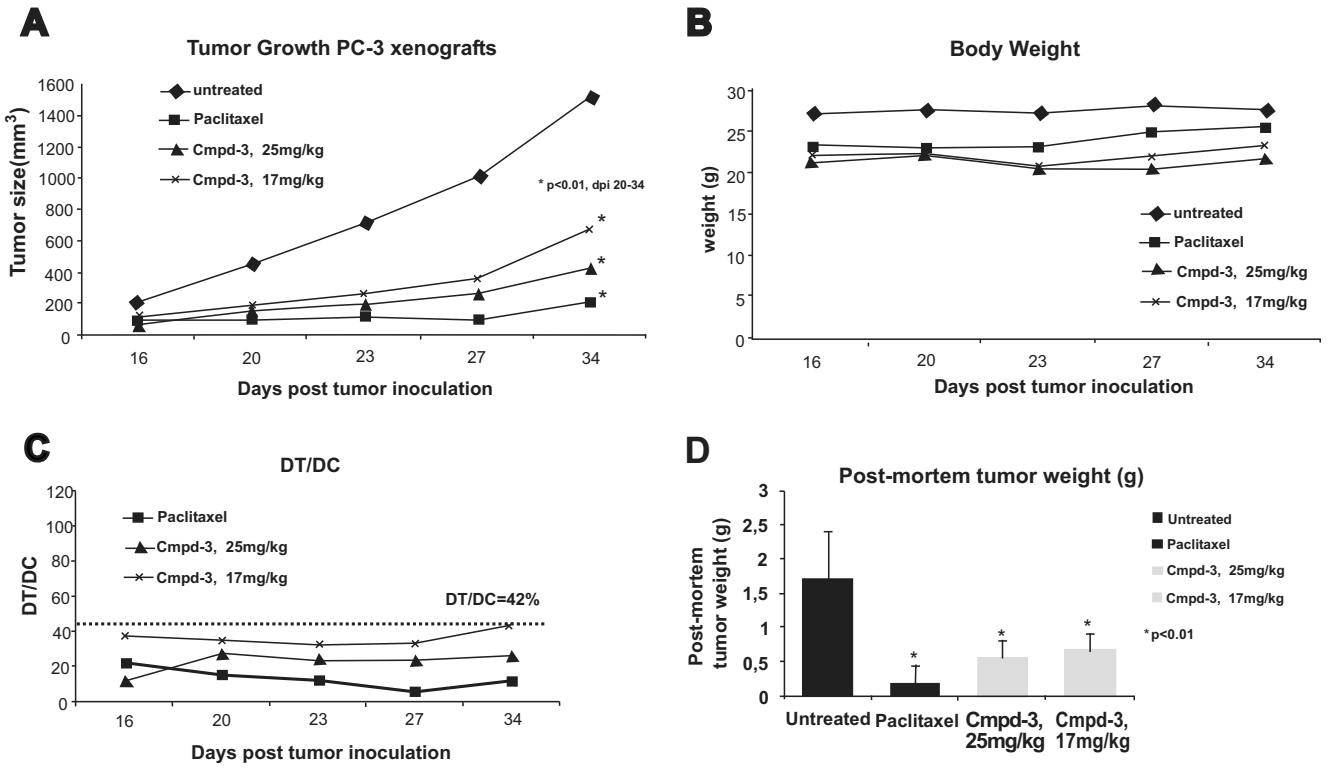

Fig. (2). Anti-cancer activity of compound 3 in PC-3 prostate cancer xenografts. Panel A: Tumor size measurements of animals treated with two doses of compound **3** (25 and 17 mg/kg) versus paclitaxel and untreated control. * indicates dose dependent statistically significant anti-cancer activity versus control (student's t-test, $p<0.01$, between dpi 20-34). Panel B. Body weight measurements of the mice treated by different compounds as described in panel A. Panel C. DT/DC measurements for compound **3** and paclitaxel calculated as described in Materials and Methods. The line denotes NCI recommended limits of significant anti-cancer activity (DT/DC < 42%). Panel D. Tumor weights excised after animal sacrifice. The results show significant anti-cancer activity for both doses of compound **3** (student's t-test, $p<0.01$).

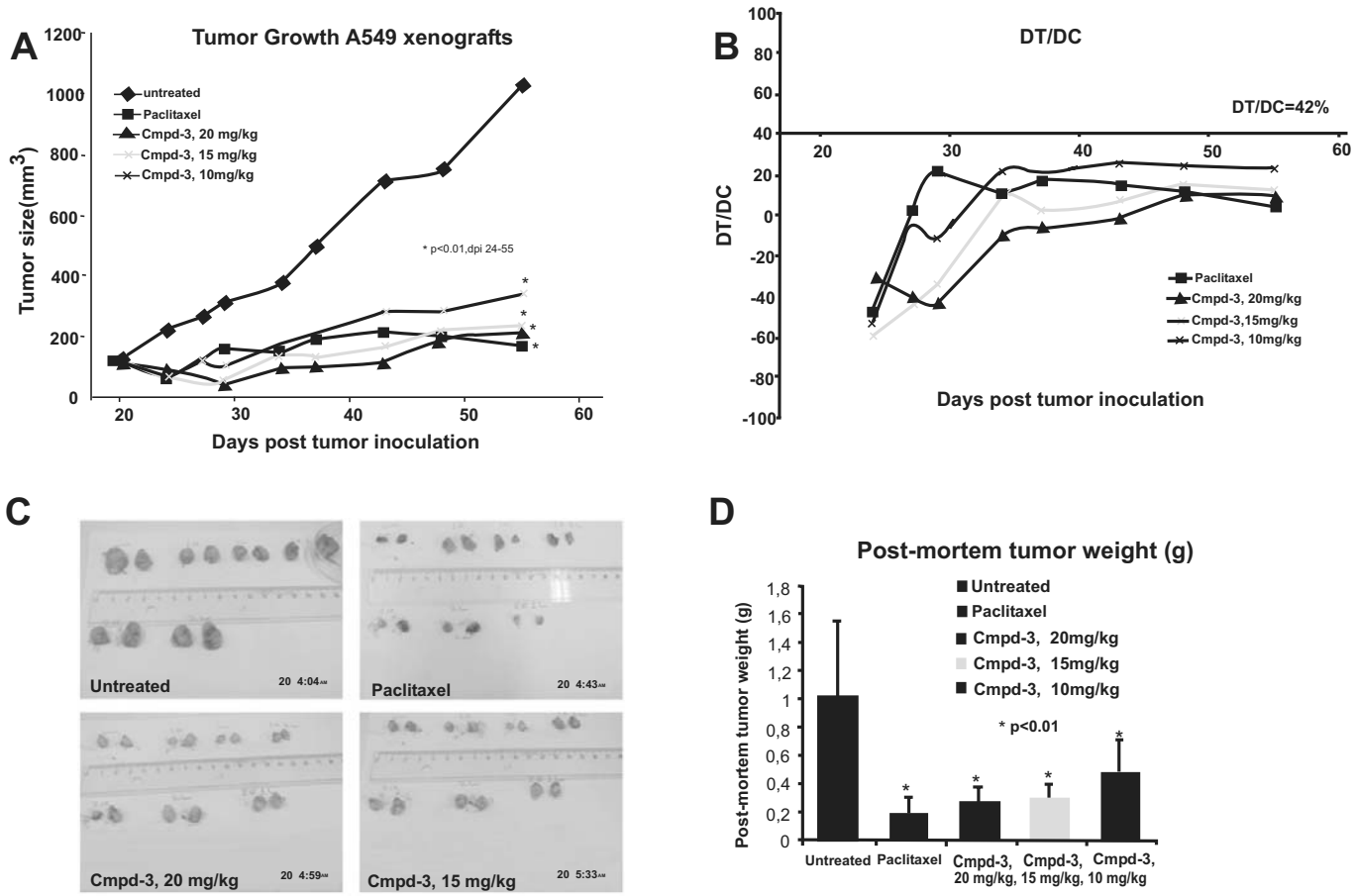

Fig. (3). Anti-cancer activity of compound 3 in A549 lung cancer xenografts. Panel A: Tumor size measurements of animals treated with three doses of compound **3** (20, 15 and 10 mg/kg) versus paclitaxel and untreated control. *indicates dose dependent statistically significant anti-cancer activity versus control (student's t-test, $p<0.01$, between dpi 24-55). Panel B. DT/DC measurements for compound **3** and paclitaxel calculated as described in Supplementary Materials and Methods. The line denotes NCI recommended limits of significant anti-cancer activity (DT/DC < 42%). Negative values denote tumor regression. Panel C. Photographs from excised tumors showing the anti-cancer effects of tested compounds. Panel D. Weights of excised tumors as shown in panel C. The results show significant anti-cancer activity of all three doses of compound **3** (student's t-test, $p<0.01$).

clinically approved for non-small cell lung cancer used as a positive control in this efficacy experiment [35]. Body weights of mice remained constant during treatment indicating that treatments were well tolerated (Fig. **2**, Panel B) although 1/6 mice in the 25 mg/kg group and 1/6 mice in the paclitaxel group died during the treatment. Post-mortem analysis of tumors excised after animal sacrifice further confirmed the strong anti-cancer effects of the compound in reducing tumor weights (Fig. **2**, Panel D). Moreover, the compound was able to suppress metastases observed in front axilla and bone lymph nodes; these were developed in only a subset of the untreated animals but were totally absent from treatment groups (data not shown). In the same experiments, paclitaxel used as a positive control was highly active, in agreement with the well documented anti-cancer properties of the compound. On the contrary, digoxin injected at a dose previously shown to have anti-cancer activity in the same model (2 mg/kg) [39], resulted in death of all animals by dpi 34 in the absence of any signs of efficacy (data not shown).

In A549 xenografts, the compound's activity was profound - even at lower doses- and resulted in very significant dose dependent growth inhibition with an optimal DT/DC ratio of -59.37 observed at dpi 24 (15 mg/kg). This effect was comparable to the effect of paclitaxel (Fig. **3**, panels A, B). Post mortem analyses of excised tumor weights confirmed these results (Fig. **3**, panels C, D). Altogether, our results showed that compound **3** has potent anti-cancer activity *in vivo* and may thus be a suitable drug candidate for further evaluation in additional cancer models.

DISCUSSION

Na^+/K^+ ATPase is a well-known target of pharmaceutical intervention in cardiac patients with congestive heart failure and Na^+/K^+ ATPase inhibitors such as digoxin & digitoxin (digitalis) are widely prescribed in the clinic due to their positive inotropic effects despite non-optimal pharmacological profiles and strong pro-arrhythmic potential [40, 27]. Recent chemical screening and epidemiological data with digitalis compounds point to a strong anti-cancer potential of these inhibitors in various cancer indications such as breast, prostate cancer and leukemia/lymphoma [22-24, 39, 41, 42] Based on these results, Na^+/K^+ ATPase inhibitors are emerging as promising new anti-cancer drugs. Of those in development, plant-derived, sugar-containing inhibitors such as UNBS1450 [43] and PBI-05204/oleandrin [44] are the most advanced compounds having shown promising preclinical activities, and in the case of PBI-05204/oleandrin, some indication of activity in phase I clinical trials [45, 15, 46, 43, 17]. A variety of cancer relevant targets such as Myc, NF-kB, HIF-1 and others modified upon addition of inhibitory compounds have also been reported (reviewed in [25]).

Steroidal Na^+/K^+ ATPase inhibitors that do not contain sugar residues constitute a novel class of Na^+/K^+ ATPase inhibitors that

may offer better development profiles in comparison to existing compounds. Based on these properties, we set out to determine whether steroidal Na^+/K^+ ATPase inhibitors with inotropic activity described previously [28-31] could possess anti-cancer activity *in vitro*. To do so, we synthesized a total of 17 steroidal inhibitors and tested them for the first time in a series of 12 cancer cell lines from 7 cancer panels. Comparing Na^+/K^+ ATPase IC_{50} activities *in vitro*, we were able to identify potent enzyme inhibitors with strong activity in most cell lines (Supplementary Tables **2** and **3**). With the exception of compound 2 whose low Na^+/K^+ ATPase IC_{50} did not translate to high anti-cancer activity for some unknown reason, compound inhibitory potencies on the Na^+/K^+ enzyme correlated well to observed anti-cancer activities. Overall, it was noted that (I) the presence of a cyclic amine in the oximic chain at position 3 was synonymous to potent enzyme inhibition and strong anti-cancer activity and (II) 6-methylene, 6-methoxyimino and 6α methanol substituents on position 6 contributed these effects. This was in agreement with previously published results, at least for what concerns Na^+/K^+ ATPase inhibition [31]. Among all compounds, 3-[(*R*)-3-pyrrolidinyl]oxime derivative **3** (Fig. **1A**) had the best activity showing outstanding potencies (as measured by GI_{50}, TGI and LC_{50} values) in most cells. Interestingly, compound **3**: (I) had activity in all cancer cell lines tested, (II) was active in multi-drug resistant NCI-ADRES cells and (III) was selectively cytotoxic in cancer versus normal cells showing a therapeutic index of 31.7 (Fig. **1D**). This was in sharp contrast with digoxin which was more cytotoxic to normal than to cancer cells.

Testing compound **3** *in vivo*, we noticed that it was strongly bioavailable with a half-life of 7.52 and 4.89h for the IP and IV routes respectively (Table **1**). Although these studies were preliminary, the measured stability properties are promising as a plasma half-life of a few hours is considered optimal for the avoidance of pro-arrhythmic effects of Na^+/K^+ ATPase inhibitors [27]. In xenograft studies, compound **3**, showed dose-dependent anti-cancer activity in both prostate and lung cancer xenografts; this activity was comparable to the activity of paclitaxel, and is considered highly potent according to NCI standards (DT/DC values inferior to 42%) (Figs. **1**, **2**). Interestingly, compound **3** was also able to suppress front axilla and bone metastasis seen in PC-3 tumors (data not shown). The observed activity in lung cancer corroborates previous observations from other groups proposing Na^+/K^+ ATPase as a very prominent target in lung cancer [17]. In addition, prostate cancer was profiled as one of the best tumor indications to develop Na^+/K^+ ATPase compounds by other researchers due to the response of these cells to UNBS1450 [46]. On the contrary, digoxin was toxic in PC-3 xenograft models resulting in death of all treatment animals (data not shown). This observation coupled with the *in vitro* data mentioned before further validate the hypothesis that digitalis compounds have narrow therapeutic indices and cannot be further developed for cancer indications.

Although current research in the Na^+/K^+ ATPase anti-cancer field has increased in the recent years, the development of novel Na^+/K^+ ATPase inhibitors as pharmaceutical agents has been impeded by: (I) lack of response biomarkers *in vivo*, (II) the requirement of performing cardiotoxic measurements using embryonic cardiac myocytes and/or telemetry in animal models and (III) in the case of novel plant-derived compounds such as UNBS1450 and PBI-05204/oleandrin, of high isolation and hemi-synthesis costs. Further lack of information regarding the composition of cardiac Na^+/K^+ ATPase complexes versus cancer Na^+/K^+ ATPase complexes as well as the existence of multiple signaling complexes containing the Na^+/K^+ ATPase (see Introduction) further complicate research activities in this area. Addressing some of the aforementioned concerns, steroidal Na^+/K^+ ATPase inhibitors offer ease of synthesis, low cost-of-goods and potentially better safety profiles. These properties, coupled with strong anti-cancer actions shown in this study promote their potential use as novel candidate anti-cancer drugs. Currently focusing our research on elucidating key mechanism of action aspects of the characterized inhibitors, we are also seeking to identify and test novel steroidal compounds with superior therapeutic indices. Pending execution of these studies, compound **3** characterized in our studies, may constitute a promising re-purposing candidate for further anti-cancer drug development.

AUTHORS' CONTRIBUTIONS

Design of research/study: K. Alevizopoulos, T. Calogeropoulou, C. Stournaras, Development of chemical methodology and synthesis: C. Baskakis, KC. Prousis, M. Tsakos, T. Calogeropoulou, Performed research/study: K. Dimas, N. Papadopoulou, S. Honisch, S. Alkahtani, Collection, analysis and interpretation of data: K. Dimas, N. Papadopoulou, S. Alkahtani, Writing of the manuscript: F. Lang, K. Alevizopoulos, C. Stournaras, Study supervision: K. Alevizopoulos, C. Stournaras

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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