Steroidal Cardiac Na\textsuperscript{+}/K\textsuperscript{+} ATPase Inhibitors Exhibit Strong Anti-Cancer Potential \textit{in vitro} and in Prostate and Lung Cancer Xenografts \textit{in vivo}

Konstantinos Dimas\textsuperscript{1}, Natalia Papadopoulou\textsuperscript{2,6}, Constantinos Baskakis\textsuperscript{3}, Kyriakos C. Prousis\textsuperscript{3}, Michail Tsakos\textsuperscript{3}, Saad Alkahtani\textsuperscript{4}, Sabina Honisch\textsuperscript{5}, Florian Lang\textsuperscript{5}, Theodora Calogeropoulou\textsuperscript{1}, Konstantinos Alevizopoulos\textsuperscript{2,6,*} and Christos Stournaras\textsuperscript{2,5,*}

\textsuperscript{1}Laboratory of Pharmacology, Faculty of Medicine, University of Thessaly, Larissa, Greece; \textsuperscript{2}Department of Biochemistry, University of Crete Medical School, Heraklion, Greece; \textsuperscript{3}Institute of Biology, Medicinal Chemistry and Biotechnology, National Hellenic Research Foundation, Athens, Greece; \textsuperscript{4}Department of Zoology, Science College, King Saud University, Riyadh, Saudi Arabia; \textsuperscript{5}Department of Physiology, University of Tübingen, Germany; \textsuperscript{6}Medexis SA, Kryoneri, Attica, Greece

Abstract: Sodium potassium pump (Na\textsuperscript{+}/K\textsuperscript{+} 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\textsuperscript{+}/K\textsuperscript{+} 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 \textit{in vitro} and \textit{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-(l)-3-pyrrolidinyl]oxime derivative 3, showed outstanding potencies (as measured by GI\textsubscript{50} and LC\textsubscript{50} values) in most cells \textit{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 \textit{in vivo}. Collectively, our results suggest that previously described cardiac Na\textsuperscript{+}/K\textsuperscript{+} ATPase inhibitors have potent anti-cancer actions and may thus constitute strong re-purposing candidates for further cancer drug development.

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

INTRODUCTION

The sodium potassium pump (Na\textsuperscript{+}/K\textsuperscript{+} ATPase) is a transmembrane protein complex composed of multiple isoform combinations of catalytic \(\alpha\), regulatory \(\beta\) and modulatory \(\gamma\) subunits acting as a key energy driver maintaining ionic and osmotic balance in cells (reviewed in [1]). Na\textsuperscript{+}/K\textsuperscript{+} 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\textsuperscript{+}/K\textsuperscript{+} ATPase inhibitors for the treatment of congestive heart failure (reviewed in [2]). Although the ion transport function of the Na\textsuperscript{+}/K\textsuperscript{+} ATPase is well documented, recent evidence points to a multi-factorial role of the enzyme in cells. For example, Na\textsuperscript{+}/K\textsuperscript{+} 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\textsuperscript{+}/K\textsuperscript{+} ATPase has been implicated in diverse cellular functions including adhesion, motility and actin dynamics [6-10]. Most recently, Na\textsuperscript{+}/K\textsuperscript{+} 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 \(\alpha1\) isoform, increase in the ratio of \(\alpha3\) to \(\alpha1\) isoforms and down regulation of \(\beta1\) isoforms have been reported in cancer cells, pointing to a complex regulation of Na\textsuperscript{+}/K\textsuperscript{+} ATPase holoenzyme formation in different cells (reviewed in [21]).

A series of additional experimental and other data further validate Na\textsuperscript{+}/K\textsuperscript{+} 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 digitoxin-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\textsuperscript{+}/K\textsuperscript{+} 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.
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; 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\(^{-4}\)M (in triplicates). IC\(_{50}\) values were calculated using the Origin\(^{\text{\textregistered}}\) 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/vclsp.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 aspirated 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\(^{\text{\textregistered}}\) 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 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 Mediclon 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 intraperitoneal 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, 2 h, 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\(_{0-\infty}\), AUC\(_{0-t}\)), elimination half-life (T\(_{1/2}\)), maximum plasma concentration (C\(_{max}\)), time to reach maximum plasma concentration (T\(_{max}\)), clearance (CL), and volume of distribution (V\(_{d}\)) 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:

\[ F = \frac{AUC_{D0-\infty} \times \text{Dose}_{IV}}{AUC_{D0-\infty} \times \text{Dose}_{(p)}} \times 100\% \]

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

**Xenograft Studies**

Xenografts were generated by subcutaneously injecting exponentially growing cultures of \( \sim 10^6 \) 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 [(axb/2)/2, where \( a=\text{length} \) and \( b=\text{width} \) 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 tumors sizes in untreated animals reached a volume of \( \sim 1000-1500 \text{ mm}^3 \). 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).
- **Groups 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
Cardiac Sodium Potassium Pump Inhibitors in Tumors

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 GI50, TGI and LC50 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 (LC50) 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 LC50 values in all cancer cell lines tested were 31.7 times lower than the LC50 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.
(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$_{0-\infty}$ | AUC$_{(0-t)}$ | MRT$_{(0-\infty)}$ | t$_{1/2}$ | T$_{max}$ | V$_z$ | CL | C$_{max}$ | F* |
| 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 (0-4): Area under the curve from the time of dosing to infinity; 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)/t$_{1/2}$; 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).
Altogether, our results showed that compound 3 as a positive control was highly active, in agreement with the well-tolerated activity of the compound in reducing tumor weights (Fig. 2). The compound was able to suppress metastases observed in preclinical models and resulted in very significant dose-dependent anti-cancer activity in vivo and in vitro. In A549 xenografts, the compound’s activity was profound—e.g., at lower doses—resulting in very significant 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 IC50 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 IC50 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 6x 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 bioavailable with a half-life of 7.52 and 4.89h for the IP and IV routes respectively (Table 3)). 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 [32].

In stark contrast with digoxin, which was more 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 [32]. 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.

On the contrary, digoxin was toxic in PC-3 xenograft models researchers due to the response of these cells to UNBS1450 [46].

To do so, we synthesized a total of 17 steroidal inhibitors and an R&D grant to KD, CS and TC by Medexis SA.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

We would like to thank Prof. C. Demetzos (Pharmacy Department, University of Athens) for providing access to animal facilities and Lejla Subasic and Tanja Loch (Department of Physiology, University of Tubingen) for the careful preparation of the manuscript. This work was supported from the University of Crete Research Committee (grants KA1562 and KA3452 to CS), the Deanship of Scientific Research at King Saud University (Research Group Project No. RGPPVPP-018 to SA and CS), the Deutsche Forschungsgemeinschaft (GRK 1302; SFB773; Mercator program to FL and CS) and an R&D grant to KD, CS and TC by Medexis SA.

**SUPPLEMENTARY MATERIAL**

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

**REFERENCES**


