

# Preclinical testing of 5-amino-1-((1*R*,2*S*,3*S*,4*R*)-2,3-dihydroxy-4-methylcyclopentyl)-1*H*-imidazole-4-carboxamide: a potent protein kinase C- $\iota$ inhibitor as a potential prostate carcinoma therapeutic

André H. Apostolatos<sup>a,\*</sup>, Christopher A. Apostolatos<sup>a,\*</sup>,  
Wishrawana S. Ratnayake<sup>a</sup>, Anthony Neuger<sup>c</sup>, Samer Sansil<sup>c</sup>,  
Marie Bourgeois<sup>b</sup> and Mildred Acevedo-Duncan<sup>a</sup>

Protein kinase C- $\iota$  (*PKC- $\iota$* ) is an oncogene overexpressed in many cancer cells including prostate, breast, ovarian, melanoma, and glioma. Previous in-vitro studies have shown that 5-amino-1-((1*R*,2*S*,3*S*,4*R*)-2,3-dihydroxy-4-methylcyclopentyl)-1*H*-imidazole-4-carboxamide (*ICA-1s*), a *PKC- $\iota$*  specific inhibitor, is effective against some cancer cell lines by decreasing cell growth and inducing apoptosis. To assess *ICA-1s* as a possible therapeutic, in-vivo studies using a murine model were performed. *ICA-1s* was tested for stability in blood serum and results demonstrated that *ICA-1s* was stable in human plasma at 25 and 37°C over a course of 2 h. Toxicity of *ICA-1s* was tested for both acute and subacute exposure. The acute exposure showed patient surviving after 48 h of doses ranging from 5 to 5000 mg/kg. Subacute tests exposed the patients to 14 days of treatment and were followed by serum and tissue collection. Aspartate aminotransferase, alkaline phosphatase,  $\gamma$ -glutamyl transpeptidase, troponin, and C-reactive protein serum levels were measured to assess organ function. *ICA-1s* in plasma serum was measured over the course of 24 h for both oral and intravenous treatments. Heart, liver, kidney, and brain tissues were analyzed for accumulation of *ICA-1s*. Finally,

athymic nude mice were xenografted with DU-145 prostate cancer cells. After tumors reached ~ 0.2 cm<sup>2</sup>, they were either treated with *ICA-1s* or left as control and measured for 30 days or until the tumor reached 2 cm<sup>2</sup>. Results showed tumors in treated mice grew at almost half the rate as untreated tumors, showing a significant reduction in growth. In conclusion, *ICA-1s* is stable, shows low toxicity, and is a potential therapeutic for prostate carcinoma tumors. *Anti-Cancer Drugs* 30:65–71 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.

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<sup>a</sup>Department of Chemistry, <sup>b</sup>College of Public Health, University of South Florida and <sup>c</sup>Translational Research Core Facility, The H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA

Correspondence to Mildred Acevedo-Duncan, PhD, Research Professor, University of South Florida, 4202 E. Fowler Ave., CHE 205 Tampa, FL 33620, USA  
Tel: +1 813 748 4715; fax: +1 813 974 3203; e-mail: macevedo@usf.edu

\*André H. Apostolatos and Christopher A. Apostolatos contributed equally to the writing of this article.

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## Introduction

*PKC- $\iota$* , an oncogene, has been shown to be overexpressed in multiple cancers including prostate, breast, ovarian, melanoma and glioma [1–5]. Loss or mutation of phosphatase and tensin homolog (*PTEN*) and mutation or overactivation of phosphatidylinositol 3-kinase (*PI3K*) occur in high frequency across many cancers [6]. Either of these conditions can lead to the overexpression of *PKC- $\iota$*  [7]. Malignant carcinoma cells overexpressing *PKC- $\iota$*  have shown resistance to T-cell-mediated apoptosis [8]. This is thought to occur by the *NF- $\kappa$ B* pathway, as *PKC- $\iota$*  is shown to help translocate *NF- $\kappa$ B* to the nucleus [9]. There is also evidence that *PKC- $\iota$*

associates with vimentin, playing a role in the epithelial to mesenchymal transition in malignant melanoma [3]. *PKC- $\iota$*  has previously been suggested as a therapeutic target for cancer patients and gold containing *PKC- $\iota$*  inhibitors were tested [10]. While therapeutics containing gold have been in use for decades, they also have adverse effects and may not be suitable for many cancer patients [11]. The effectiveness of the nucleotide analog of *ICA-1s* has been previously tested *in-vitro* on malignant neuroblastoma and glioma cells lines overexpressing *PKC- $\iota$* . These studies have shown that *ICA-1s* is effective against some cancer cell lines, with a decrease in malignant cell growth and induction of apoptosis. These studies have also shown that *ICA-1s* is specific to *PKC- $\iota$* , leaving normal cells that do not overexpress *PKC- $\iota$*  largely unaffected [1,3]. While the efficacy of *ICA-1s* is well documented *in-vitro*, it has not been tested *in-vivo* [1,3,12]. To determine whether *ICA-1s* could be a potential

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alternative therapy for some cancer patients, it was necessary to test key factors. This study tested ICA-1s stability, toxicity, pharmacokinetics, and therapeutic efficacy on in-vivo patients. The results show further investigation into ICA-1s as a therapeutic is warranted to assess the potential for progress toward clinical trials.

## Materials and methods

### Cell culturing

RWPE-1 (ATCC CRL-11609) epithelial cells and DU-145 (ATCC HTB-81) human prostate carcinoma cells were purchased from American Type Culture Collection (Manassas, Virginia, USA). PC-3 cells were acquired from Moffitt Cancer center (Tampa, Florida, USA). The cells were grown as a monolayer in a T25 tissue culture flask with 5 ml of growth medium and were maintained in a 37°C incubator with 5% CO<sub>2</sub>. The E-MEM and F-12 growth media were obtained from American Type Culture Collection. The medium was supplemented with 10% fetal bovine serum and a mix of the antibiotics penicillin (10 000 IU) and streptomycin (10 000 µg/ml) in a 100× concentration purchased from Corning (Corning, New York, USA). Both athymic and wild-type mice were ordered from Jackson Laboratories (Bar Harbor, Maine, USA). The protocol was approved by the University of South Florida under IACUC protocol no.: R IS00001888.

### Nuclear magnetic resonance

Structure of the ICA-1s was confirmed by Innova400-1 NMR spectrophotometer (Varian Inc., Palo Alto, California, USA) using DMSO-d<sub>6</sub> as the solvent (30 mmol/l).

### WST-1 assay for cell viability and cytotoxicity

WST-1 assay (*in-vitro*) was performed by culturing  $\sim 3.5 \times 10^3$  cells/well (RWPE-1, DU-145, and PC-3 cells) in a 96-well plate. After 24 h postplating time, fresh media were supplied (200 µl/well) and treated with either an equal volume of sterile water (vehicle control) or with 23.4 mmol/l of ICA-1s. The applied ICA-1s concentration (23.4 mmol/l) was determined as the equivalent in-vitro concentration considering the highest tested in-vivo concentration (200 mg/kg) for an average volume of the blood of a mouse (1.0 ml) and an average weight of a mouse (30.0 g). Additional doses were supplied every 48 h during a 4 day incubation period. At the end of day 4, media were removed and fresh media (100 µl) were added with 4-[3-(4-iodophenyl)-2-(4-nitrophenyl))-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) reagent (10 µl) to each well. The absorbance was measured at 450 nm for every 1 h up to 8 h using the Synergy HT microplate reader from Biotek (Winooski, Vermont, USA).

### Acute toxicity

An oral up and down procedure (UDP) was used to determine median lethal dose (LD<sub>50</sub>) and one animal was tested at a time, and the response of each animal to a test

substance determines whether the next animal receives a higher or lower dose.

### Dose-response

For establishing the dose-response of ICA-1s (all test substances administered intravenous): group 1: vehicle control; group 2: ICA-1s (50 mg/kg); group 3: ICA-1s (100 mg/kg); group 4: ICA-1s (200 mg/kg). Six animals were in each group. Animals were euthanized by placement in a carbon dioxide chamber at 6 or 18 h post-ICA-1s administration, after which they sustained cervical dislocation as a secondary means of euthanasia. Blood and tissue were collected. Functional perturbation of the liver, kidneys and heart were tested by measuring serum levels of the enzymes aspartate aminotransferase (AST) (catalog number #K753-100 from BioVision Inc., Milpitas, California, USA),  $\gamma$ -glutamyl transpeptidase (GGT) (catalog number #K784-100 from BioVision Inc.), C-reactive protein (CRP) (catalog number #EK294 from BioOcean, Shoreview, Minnesota, USA), and troponin (catalog number #ABIN1117615 from Elabscience Biotechnology Inc., Houston, Texas, USA). Tissues collected were assessed for gross pathology and morphology.

### Plasma stability and murine drug quantitation (pharmacokinetics and bioavailability)

Plasma stability was first evaluated as a component of general compound evaluation and in order to ensure no special processing needs were required for mouse blood and tissue sampling. This was performed in both mouse and human plasma. Samples spiked with drug were measured after separate incubations of 25 and 37°C. Plasma levels were measured after both intravenous and oral dosing for typical pharmacokinetic and bioavailability studies to evaluate compound half-life and other typical characteristics that require an evaluation of a drug candidate. Tissue levels of several organs of interest were also evaluated to look at the biodistribution of the compound and to determine if any accumulation of compound in any one particular organ or organs were witnessed. The following organs were collected during the sacrifice procedures: liver, kidney, heart and brain.

### Liquid chromatography-mass spectrometry methodology

To form calibrations sets for plasma analysis, mouse plasma spiked with known concentrations of unphosphorylated ICA-1s was added to a Sirocco (Waters Corporation, Milford, Massachusetts, USA) protein precipitation plate containing a solvent consisting of acetonitrile and methanol. The plate was vortexed briefly and then placed in a centrifuge (Eppendorf, Hamburg, Germany) to stimulate the pass-through of the desired eluent into a collection plate. The eluent is then evaporated with an Ultravap system (Porvair Sciences, Wrexham, UK). Samples are reconstituted with 0.1% acetic acid and vortexed before instrument analysis.

Unknown mouse plasma samples collected from the experimental sets were treated as described above [13].

To form calibration for tissue studies, untreated (blank) tissue of each organ matrix was spiked with known concentrations of ICA-1s and homogenized in ice-cold 0.1% acetic acid at a concentration of 10 ml/g. After centrifugation, a solvent consisting of acetonitrile and methanol was added to the supernatant and the samples are vortexed, centrifuged, and evaporated after centrifugation. Samples were reconstituted with 0.1% acetic acid and vortexed before instrument analysis. Unknown mouse tissue samples collected from the experimental sets were treated similarly as above [13].

The sample was injected into a Thermo Accela Ultra-High Performance Liquid Chromatography system coupled to a Thermo TSQ Quantum Tandem Mass Spectrometer (Thermo Electron, San Jose, California, USA). Gradient elution was achieved with mobile phases of water and methanol, both containing 0.1% acetic acid. A Phenomenex Luna reverse phase C18 column (Phenomenex, Torrance, California, USA) was used to separate compounds. The mass spectrometry system employs heated electrospray ionization in the source followed by selected reaction monitoring of the target compound. The following selected reaction monitoring transition was monitored in positive ion mode for quantitation: 240.100→110.054 of ICA-1s. The assay had a linear range from 5 to 2500 ng/ml for plasma and 5 to 5000 ng/ml in tissue [14].

### Xenografts

DU-145 prostate carcinoma cells were cultured and trypsonized.  $1 \times 10^6$  cells were inserted subcutaneously to the flank of athymic nude mice. Mice were separated into two groups, treated and untreated. The treated group was given 100 mg/kg of ICA-1s by subcutaneous injection daily beginning when the tumor reached a size of 0.2 cm<sup>2</sup>. Tumors size was measured daily by external digital caliper. The endpoints were reached after 30 days of measurement or if the tumor size reached 2 cm<sup>2</sup>.

## Results

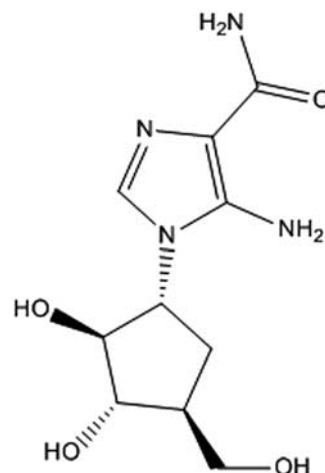
### Nuclear magnetic resonance

The structure of ICA-1s (Fig. 1) was confirmed by <sup>1</sup>H NMR:  $\delta$  1.7 (<sup>1</sup>H, ddd,  $J$ =13.9, 7.0, 5.3 Hz), 2.1–2.5 [<sup>4</sup>H, 2.15 (ddd,  $J$ =13.7, 8.1, 7.5 Hz), 2.45 (ddtd,  $J$ =9.5, 7.6, 7.4, 6.8 Hz), 2.41 (ddd,  $J$ =13.9, 9.4, 7.6 Hz), 2.21 (ddd,  $J$ =13.7, 6.8, 4.1 Hz)], 3.6–4.0 [<sup>2</sup>H, 3.72 (td,  $J$ =9.4, 5.3 Hz), 4.01 (d,  $J$ =7.4 Hz)], 4.4 (<sup>1</sup>H, dddd,  $J$ =8.1, 7.6, 7.0, 4.1 Hz), 7.55 (<sup>1</sup>H, s); <sup>13</sup>C NMR:  $\delta$  43.7, 55.6, 142.6, 130.2, 34.1, 117.0, 156.5, 38.9, 66.8, 79.1.

### WST-1 assay for cell viability and cytotoxicity

To determine the in-vitro cytotoxicity of ICA-1s (Fig. 2) on normal and malignant cell lines, we performed a WST-1 assay. The measured absorbance at 450 nm is

Fig. 1



The chemical structure of 5-amino-1-((1*R*,2*S*,3*S*,4*R*)-2,3-dihydroxy-4-methylcyclopentyl)-1*H*-imidazole-4-carboxamide (ICA-1s), the inhibitor studied in this report.

directly proportional to the number of viable cells present which produce a water-soluble formazan with WST-1 as a result of their mitochondrial dehydrogenase activity. ICA-1s did not demonstrate significant cytotoxicity on the normal prostate epithelial cell line (RWPE-1) (Fig. 2a). As shown in Fig. 2b and c, ICA-1s showed a significant cytotoxicity ( $P \leq 0.01$ ) on both metastatic prostate cancer cell lines (DU-145 and PC-3). Results indicate that the in-vitro ICA-1s concentration (23.4 mmol/l) is extremely cytotoxic to prostate cancer cells compared with the relatively low cytotoxicity posed on normal cells during the tested time range, thereby retarding prostate cancer cell growth and proliferation.

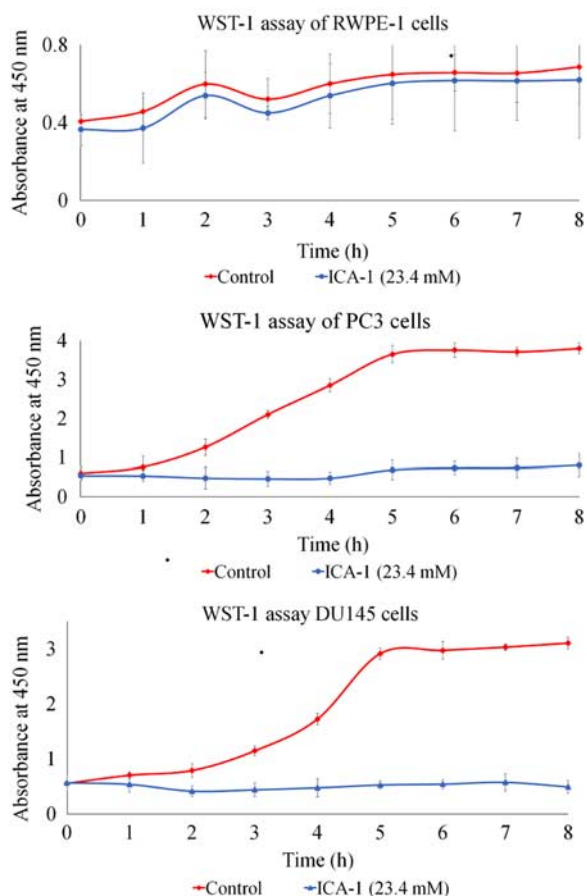
### Plasma stability and murine drug quantitation (pharmacokinetics and bioavailability)

ICA-1s was proven to be stable in human plasma over 2 h at both at ambient conditions (25°C) and at 37°C (Fig. 3).

### Spectroscopy

The compound appears to have linear kinetic behavior in mouse plasma with a half-life of 5.7 h. See Fig. 4 for a graphical representation. This was produced in both oral gavage and intravenous bolus push injection. There was a large increase in plasma concentration of the intravenous dosing versus the oral dosage. The oral dose was ~30% of the intravenous dose. This is reflected in the area under the curve, maximum concentration ( $C_{max}$ ), and volume of distribution. Compound tissue levels did not appear to indicate accumulation in any of the major organs analyzed as tissue levels approached lower limits of quantitation by 24 h. There was, however, a recirculation of compound in the heart, liver, and kidney after 12 h of intravenous administration and recirculation of compound in heart,

Fig. 2



The toxicity of 5-amino-1-((1*R*,2*S*,3*S*,4*R*)-2,3-dihydroxy-4-methylcyclopentyl)-1*H*-imidazole-4-carboxamide (ICA-1s) over 8 h at a concentration of 23.4 mmol/l. The RWPE-1 cell line is compared with malignant cell lines PC-3 and DU-145.

liver, and brain after 8 h of oral dosing, before the drug levels continuing on a path of clearance at the next sample time point.

#### Acute toxicity

The UDP began at 50 mg/kg and increased by a factor of 3.2. The tests stopped when the concentration reached 2000 mg/kg. The animal survived with no observable adverse reaction and monitoring stopped after 48 h. The test was repeated twice on other animals to verify results.

#### Dose-response

To determine alteration of organ function in levels of enzymes AST, GGT, troponin I, and CRP in blood serum were tested by colorimetric assay. Six animals were in each test group. Animals treated with ICA-1s were given an intravenous dose of either 50, 100, or 200 mg/kg. Another test group received no drug and was used as a control group. Animals were sacrificed either 6 or 18 h after treatment. After 6 h of treatment at a dose of 200 mg/kg there was a significant rise in CRP ( $P=0.033$ ). All other readings for CRP, AST, GGT, and troponin I tested showed no significant difference compared with the control.

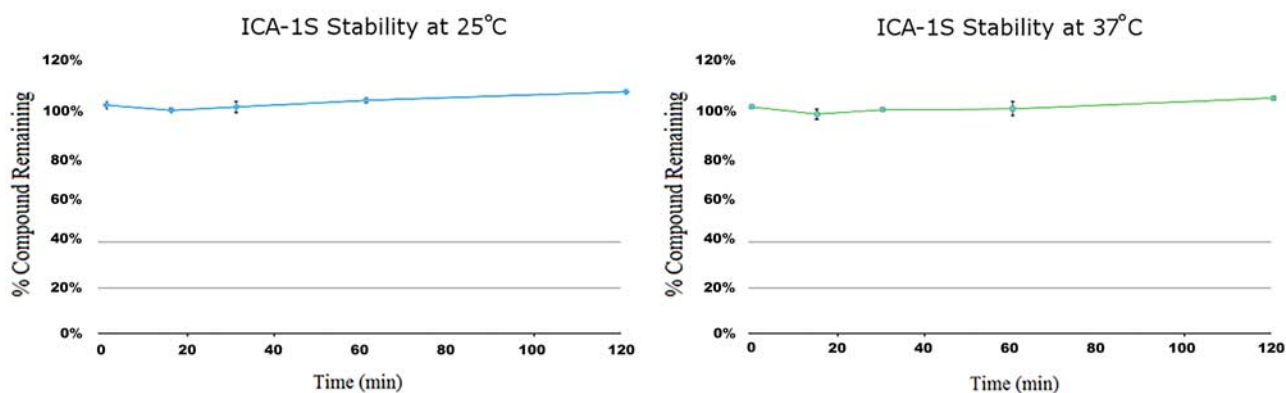
#### Xenografts

To test the effectiveness of ICA-1s *in vivo*, 12 mice were injected with DU-145 prostate carcinoma cells. The average tumor size of the treated xenografts was 76.8 mm<sup>2</sup>. The average tumor size of the untreated xenografts was 156.4 mm<sup>2</sup> (Figs. 5–7).

#### Discussion

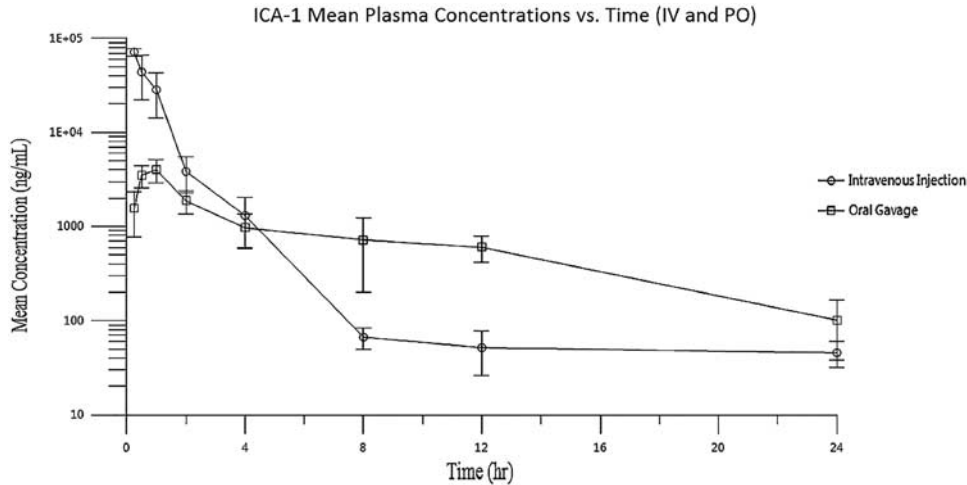
This study sought to investigate the potential of ICA-1s as a therapeutic by evaluating the effectiveness ICA-1s has against malignant tumor cells, balanced against the

Fig. 3



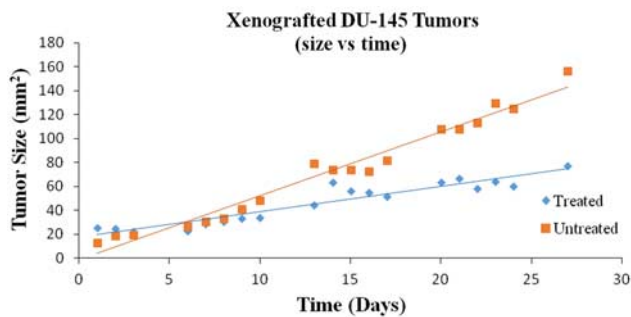
The stability of 5-amino-1-((1*R*,2*S*,3*S*,4*R*)-2,3-dihydroxy-4-methylcyclopentyl)-1*H*-imidazole-4-carboxamide (ICA-1s) over the course of 120 min at both 25 and 37°C.

Fig. 4



The plasma concentration of 5-amino-1-((1*R*,2*S*,3*S*,4*R*)-2,3-dihydroxy-4-methylcyclopentyl)-1*H*-imidazole-4-carboxamide (ICA-1s) over time with intravenous (i.v.) compared with oral dose (p.o.). The area under the curve represents the total exposure to ICA-1s.

Fig. 5



The progression of tumor growth for mice xenografted with DU-145 cells. Mice were treated with either 100 mg/kg 5-amino-1-((1*R*,2*S*,3*S*,4*R*)-2,3-dihydroxy-4-methylcyclopentyl)-1*H*-imidazole-4-carboxamide (ICA-1s) or an equivalent volume of saline. The untreated group is represented in orange. The treated group is represented in blue.

Fig. 6



A visual representation of tumors after they were excised from host animals. The top row shows untreated tumors after 30 days, while the bottom row shows tumors after 30 days of treatment with 5-amino-1-((1*R*,2*S*,3*S*,4*R*)-2,3-dihydroxy-4-methylcyclopentyl)-1*H*-imidazole-4-carboxamide (ICA-1s).

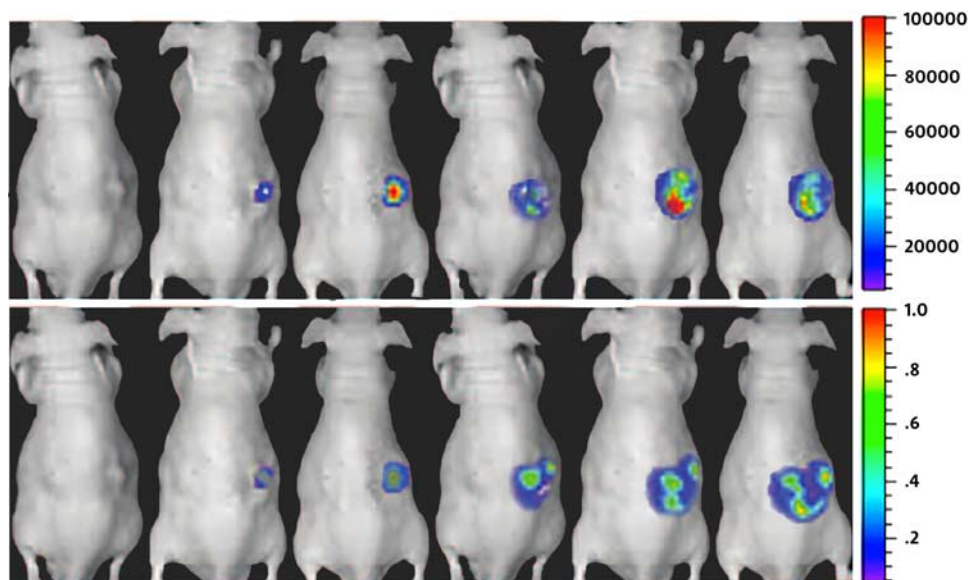
toxicity ICA-1s exerts on healthy tissue. The treatment of xenografted tumor cells showed a significant decrease in the rate of tumor growth. Notably, there was no reduction in tumor size. This suggests that while ICA-1s may retard cell proliferation, it is not directly responsible for tumor cell death. This evidence is mirrored in the WST-1 assay, which showed that ICA-1s had a significant effect on malignant prostate cancer cell growth without reducing cell numbers below their starting values. These results also suggest that *PKC- $\tau$*  plays a role in tumor cell proliferation as seen in previous studies [3,14]. This evidence also supports previous studies that show *PKC- $\tau$*  is part of an antiapoptotic pathway and its inhibition allows for cell-mediated apoptosis [8,12,15].

These results also help explain the relatively low toxicity of ICA-1s on normal tissue. The WST-1 assay showed that ICA-1s had a relatively smaller effect on RWPE-1 transformed cells compared to malignant cells. In addition, the UDP was stopped at 2000 mg/kg without producing a lethal response. The enzyme markers showed no damage to the heart or liver confirming the gross anatomy inspection. The highest treated dose of 200 mg/kg produced a moderate increase in CRP, a marker for inflammation, at the 6 h mark, but levels were back to baseline by the 18 h mark. These results point to a very specific inhibitor that does not have a toxic effect on normal tissue.

As a pharmaceutical, ICA-1s has qualities that warrant further exploration into its potential. Results showed that it



Fig. 7



The bioluminescent monitoring of prostate cancer tumor establishment on mouse models from the superior position. Images were taken postinjection and weekly thereafter. These represent a typical growth pattern observed in xenografted mice.

was stable at both 25 and 37°C for 2 h. Furthermore, there was no accumulation in any of the organs tested. The recirculation of ICA-1s at the 12 h mark may have been variability of the experiment itself due to a requirement of an individual sacrifice per time point, but could also be an item to revisit. Because of much higher concentration levels in intravenous dosing coupled with the recirculation of compound in several sites in series, it is estimated that first-pass elimination (or presystemic metabolism) is in effect. ICA-1s may depend on physiological factors such as enzyme activity, plasma protein and blood-cell binding, and gastrointestinal motility [16]. The half-life 5.7 h is in similar to some other pharmaceuticals already used to treat prostate cancer such as flutamide [17].

Currently, there are a wide variety of treatments for prostate cancer. Treatments like radiation therapy risk exposing the patient to the subsequent bladder and rectal cancer [18]. Age is an important risk factor for post-operative mortality [19]. This means surgery typically carries higher risks for prostate cancer patients as the average age of discovering prostate cancer is 66 years old [20]. Furthermore, previous studies have shown that a prostatectomy may not improve a patient survival rate compared with observation [21]. As a result, active surveillance is adopted in many patients. As some prostate tumors are slow-growing some patients never require intervention. As a potential therapeutic ICA-1s may be of particular benefit to patients with slow-growing tumors. If treatment with ICA-1s can further diminish the progression of prostate cancer tumors, it would allow patients to avoid risks associated with surgery or radiation therapy.

As ICA-1s does not target antiandrogens like some therapeutics currently in use [22], it may also have value as part of a regimen that uses a combination of drugs. Given the pharmacokinetic properties previously described, ICA-1s could potentially be used as an oral therapeutic either with or in place of some therapeutics currently in use.

Some of the limitations of this study included the sample size, length of observation, and a variety of tumor sources. We suggest further studies to address some of the limitations of this study. We also suggest further investigation into ICA-1s derivatives for potentially more potent candidates.

Cumulatively our results indicate ICA-1s is inhibitor specific to the oncogenic protein *PKC- $\alpha$* , and use of ICA-1s leads to a significant reduction in prostate cancer growth rate while leaving healthy tissue relatively unaffected. This means that ICA-1s could be a very useful agent against prostate cancer and further efforts should be made to develop the compound for clinical use.

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### Conflicts of interest

There are no conflicts of interest.

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