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ORIGINAL RESEARCH

Cellular Mechanisms Underlying the Low Cardiotoxicity of Istaroxime

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BACKGROUND: Istaroxime is an inhibitor of Na^+/K^+ ATPase with proven efficacy to increase cardiac contractility and to accelerate relaxation attributable to a relief in phospholamban-dependent inhibition of the sarcoplasmic reticulum Ca^{2+} ATPase. We have previously shown that pharmacologic Na^+/K^+ ATPase inhibition promotes calcium/calmodulin-dependent kinase II activation, which mediates both cardiomyocyte death and arrhythmias. Here, we aim to compare the cardiotoxic effects promoted by classic pharmacologic Na^+/K^+ ATPase inhibition versus istaroxime.

METHODS AND RESULTS: Ventricular cardiomyocytes were treated with ouabain or istaroxime at previously tested equi-inotropic concentrations to compare their impact on cell viability, apoptosis, and calcium/calmodulin-dependent kinase II activation. In contrast to ouabain, istaroxime neither promoted calcium/calmodulin-dependent kinase II activation nor cardiomyocyte death. In addition, we explored the differential behavior promoted by ouabain and istaroxime on spontaneous diastolic Ca^{2+} release. In rat cardiomyocytes, istaroxime did not significantly increase Ca^{2+} spark and wave frequency but increased the proportion of aborted Ca^{2+} waves. Further insight was provided by studying cardiomyocytes from mice that do not express phospholamban. In this model, the lower Ca^{2+} wave incidence observed with istaroxime remains present, suggesting that istaroxime-dependent relief on phospholamban-dependent sarcoplasmic reticulum Ca^{2+} ATPase 2A inhibition is not the unique mechanism underlying the low arrhythmogenic profile of this drug.

CONCLUSIONS: Our results indicate that, different from ouabain, istaroxime can reach a significant inotropic effect without leading to calcium/calmodulin-dependent kinase II–dependent cardiomyocyte death. Additionally, we provide novel insights regarding the low arrhythmogenic impact of istaroxime on cardiac Ca^{2+} handling.

Key Words: Ca^{2+}/calmodulin-dependent kinase II ■ cardiotoxicity ■ digitalis and apoptosis ■ istaroxime

Pharmacologic blockade of Na^+/K^+ ATPase (NKA) with digitalis has been extensively used as a therapy for patients suffering heart failure because of its positive inotropic effect.[1] However, in the past decades, the use of digitalis compounds like digoxin was strongly reduced because of their associated toxicity, which includes life-threatening arrhythmias and clinical trials not providing evidence of reduced mortality.[2] The clinical use of digoxin was maintained in patients with heart failure with atrial fibrillation because of its capacity to slow atrioventricular conduction.[3] More recently, the necessity of inotropic modulators has motivated new clinical trials with other NKA inhibitors such as digitoxin and istaroxime.[4,5]

Istaroxime is a steroidal compound not related to cardiac glycosides, which combines NKA blocking capacity with a potentiation of Ca^{2+} uptake into the sarcoplasmic reticulum (SR) mediated by the SR Ca^{2+} ATPase (SERCA) 2a and is the first inotropic and lusitropic agent shown to be effective and safe in patients.[6] This peculiar combination of targets seem to confer istaroxime a better safety profile compared with other NKA inhibitors, given that a lower risk of Ca^{2+} triggered arrhythmias was reported[7] and that

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CLINICAL PERSPECTIVE

What Is New?

- In contrast to ouabain, istaroxime can reach a significant inotropic effect without leading to Ca^{2+}/calmodulin-dependent kinase II–dependent cardiomyocyte death.
- Istaroxime does not increase Ca^{2+} spark and wave frequency but breaks Ca^{2+} waves into less arrhythmogenic mini-waves.
- The absent arrhythmogenic effect of istaroxime does not rely exclusively on sarcoplasmic reticulum Ca^{2+} ATPase/phospholamban dissociation.

What Are the Clinical Implications?

- This work provides evidence that supports the safety of istaroxime for the treatment of heart failure and for other potential uses without cardiotoxicity.

Nonstandard Abbreviations and Acronyms

CaMKII	Ca^{2+}/calmodulin-dependent kinase II
NKA	Na^+/K^+ ATPase
SERCA	sarcoplasmic reticulum Ca^{2+} ATPase
SR	sarcoplasmic reticulum

the infusion of istaroxime for 24 hours was recently shown to improve heart function in patients hospitalized for acute heart failure without major adverse events.[5]

We have previously shown that low "therapeutic" concentrations of digitalis can promote Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activation, which leads to cardiomyocyte death.[8] Additionally, we were the first to link CaMKII activation with digitalis-induced arrhythmias.[9]

Interestingly, istaroxime has been shown to have an antiproliferative capacity in tumoral cell lines[10] and, similar to other NKA inhibitors,[11,12] is proposed as an antitumoral drug. However, whether istaroxime also affects cardiomyocyte viability has not been previously reported. To explore this issue and to examine its capacity to activate CaMKII are aims of this study.

Focusing on Ca^{2+} handling, the currently accepted mechanism for the lusitropic effect and the low arrhythmogenicity of istaroxime is its capacity to dissociate SERCA from its inhibitory protein phospholamban,[13] but if this is the exclusive mechanism supporting istaroxime's improved safety has not been fully explored. In fact, the acceleration of Ca^{2+} uptake has been shown to promote rather than prevent arrhythmias attributable to SR overload.[14] In this context, an additional aim of this work is to gain insight into this mechanistic aspect by comparing the effects promoted by istaroxime and ouabain in cardiomyocytes lacking phospholamban (phospholamban-knockout myocytes).

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23, revised 2011) and approved by the Institutional Animal Care and Use Committee of La Plata University School of Medicine.

Chemicals

Istaroxime hydrochloryde (or PST2744 hydrochloride) was obtained from MedChemExpress (HY-15718A) and Ouabain from Sigma Aldrich (O3125). Control and ouabain solution were added with 0.05% dimethyl sulfoxide to account for the dissolution of istaroxime's preparation.

Ventricular Cardiac Myocyte Isolation and Culture

Male Wistar rats (200–300 g) were anesthetised by an intraperitoneal injection of urethane (1.2–1.4 g/kg), and hearts were excised when plane 3 of phase III of anesthesia was reached. Plane 3 of phase III of anesthesia was verified by the presence of slow, deep diaphragmatic breathing, loss of the corneal reflex, and the absence of tongue retraction. Mice were euthanized by cervical dislocation. Ventricular cardiomyocytes were isolated by collagenase-based enzymatic digestion as previously described.[15] In brief, hearts were mounted on a Langendorf perfusion apparatus and were perfused retrogradely at a constant flow with a HEPES-based salt solution (isolation solution; see below). When the coronary circulation had cleared of blood, perfusion was continued for 5 minutes with Ca^{2+}-free isolation solution containing 0.1 mmol/L EGTA, and then for 15 minutes with a solution containing 0.05 mmol/L $CaCl_2$, 0.5 mg/mL collagenase type II (300 U/mL), 0.025 mg/mL protease, and 1.25 mg/mL BSA at 37°C. After digestion was completed, the heart was disassembled, and the ventricular tissue was mechanically dissociated. The suspension of cells was then subjected to 4 steps of decantation and resuspension in isolation solutions with increasing concentration of $CaCl_2$, until a final concentration

of 1 mmol/L $CaCl_2$ was reached; cells were kept in suspension in this solution until use. HEPES (isolation solution in mmol/L): NaCl 146.2, KCl 4.7, $CaCl_2$ 1, HEPES 10, NaH_2PO_4. H_2O 0.35, $MgSO_4 \cdot 7H_2O$ 1.05, Glucose 10 (adjusted to pH 7.4 with NaOH).

For culture, isolated cells were resuspended in serum-free medium 199 (Thermo Fisher Scientific, Waltham, MA) supplemented with (in grams per liter) 0.017 ascorbic acid, 2 BSA, 0.4 L-carnitine, 0.66 creatine, 0.62 taurine, 50 U/mL penicillin, and 50 U/mL streptomycin. Myocytes were plated at a density was of $\approx 2 \times 10^4$ rod-shaped cells/mL into culture dishes for 1 hour to allow cell attachment. After this period, the culture medium was changed for a fresh one with or without ouabain or istaroxime. After 24 hours of culture at 37°C in 95% air and 5% CO_2, the cells were photographed to assess viability. Cells were evaluated morphologically, being classified as viable or nonviable according to their length-to-width ratio (>3 were considered viable).[8,16] From each culture, which was considered as an n=1, at least 8 photographs per group were taken to count and classify the cells. All data of cell viability are expressed as percentage of the total number of cells.[8]

Cell Shortening Measurements

Myocytes were mounted on an inverted microscope, superfused with HEPES-based solution (1 mmol/L Ca^{2+}), and paced via field stimulation using extracellular platinum electrodes at 1 Hz at room temperature. Once stability was reached between minutes 3 and 5, treatment with ouabain or istaroxime started. Cell shortening was monitored by video detection of cell length.[16]

Detection of Spontaneous Ca^{2+} Release by Confocal Microscopy

Myocytes were loaded at room temperature during 10 minutes with 10 µmol/L Fluo-4 AM (Thermo Fisher Scientific). After wash, cells were seeded on a perfusion chamber and mounted on a Zeiss 410 inverted confocal microscope (LSMTech, Wellsville, PA) using a ×63 oil immersion objective with 1.4 NA. Cells were imaged in linescan mode along their long axis, exciting with the 488 nm line of an argon laser and collecting emission at 500 to 550 nm.[9] Fluorescence was recorded in 512×512 pixels images, which were analyzed with Sparkmaster pluging for Image J (National Institutes of Health, Bethesda, MD).[17]

Immunodetection by Western Blot Analysis

Rat ventricular cardiomyocytes were incubated during 60 minutes in control, ouabain 2 µmol/L or istaroxime 10 µmol/L. Then, cells were collected and homogenized with lysis buffer. Protein was measured by the Bradford method using BSA as standard. Lysates (≈90 µg of total protein per gel line) were seeded in a 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Blots were probed overnight with antibodies raised against oxidized CaMKII (Ox-CaMKII, 1:1000; Millipore Corp, Billerica, MA), phospholamban (1:1000; Badrillal Leeds, UK) and Anti Bax (Bax, 1:500; Abcam, Cambridge, MA). After stripping the blots were probed with phospho-Thr286-CaMKII (1:1000; Abcam) phospho-Thr17- phospholamban (p-Thr17 phospholamban, 2:500; Badrilla), Bcl-2 (Bcl-2, 1:1000; Santa Cruz Biotechnology, Dallas, TX) and anti-GAPDH (GAPDH, 1:10000; Santa Cruz Biotechnology) was used as loading control for normalization.

Immunoreactivity was visualized by a peroxidase-based chemiluminescence detection kit (Amersham Biosciences, Amersham, UK) using a Chemidoc Imaging System (Bio-Rad Laboratories, Hercules, CA). The signal intensity of the bands in the immunoblots was quantified by densitometry using Image J software.[18]

Statistical Analysis

Unpaired Student *t* test, and 1-way ANOVA followed by Tukey's posttest were used for statistical comparisons of quantitative data. Fisher's exact test was used to compare the incidence of qualitative data between groups. Statistical analyses were performed with Prism 6.0 (GraphPad Software, La Jolla, CA). Data are expressed as mean±SEM. Differences were considered significant at $P<0.05$.

RESULTS

Determination of Equi-Inotropic Concentrations of Ouabain and Istaroxime

To compare the toxic effects of istaroxime and ouabain, we determined the equi-inotropic concentrations of both compounds by superfusing rat ventricular myocytes with the mentioned drugs and measuring cell shortening. We have previously used ouabain 2 µmol/L as a therapeutic concentration in rodent cardiomyocytes,[8] while istaroxime 4 and 10 µmol/L have been reported as equivalent of therapeutic concentrations in mice.[7] Figure 1A shows typical recordings of cell shortening before and after treatment with the mentioned drugs. After a first trial where cells treated with istaroxime 4 µmol/L showed a positive inotropic effect of 8.1±4.9% (n=9), we determined that 2 µmol/L ouabain and 10 µmol/L istaroxime both promote an

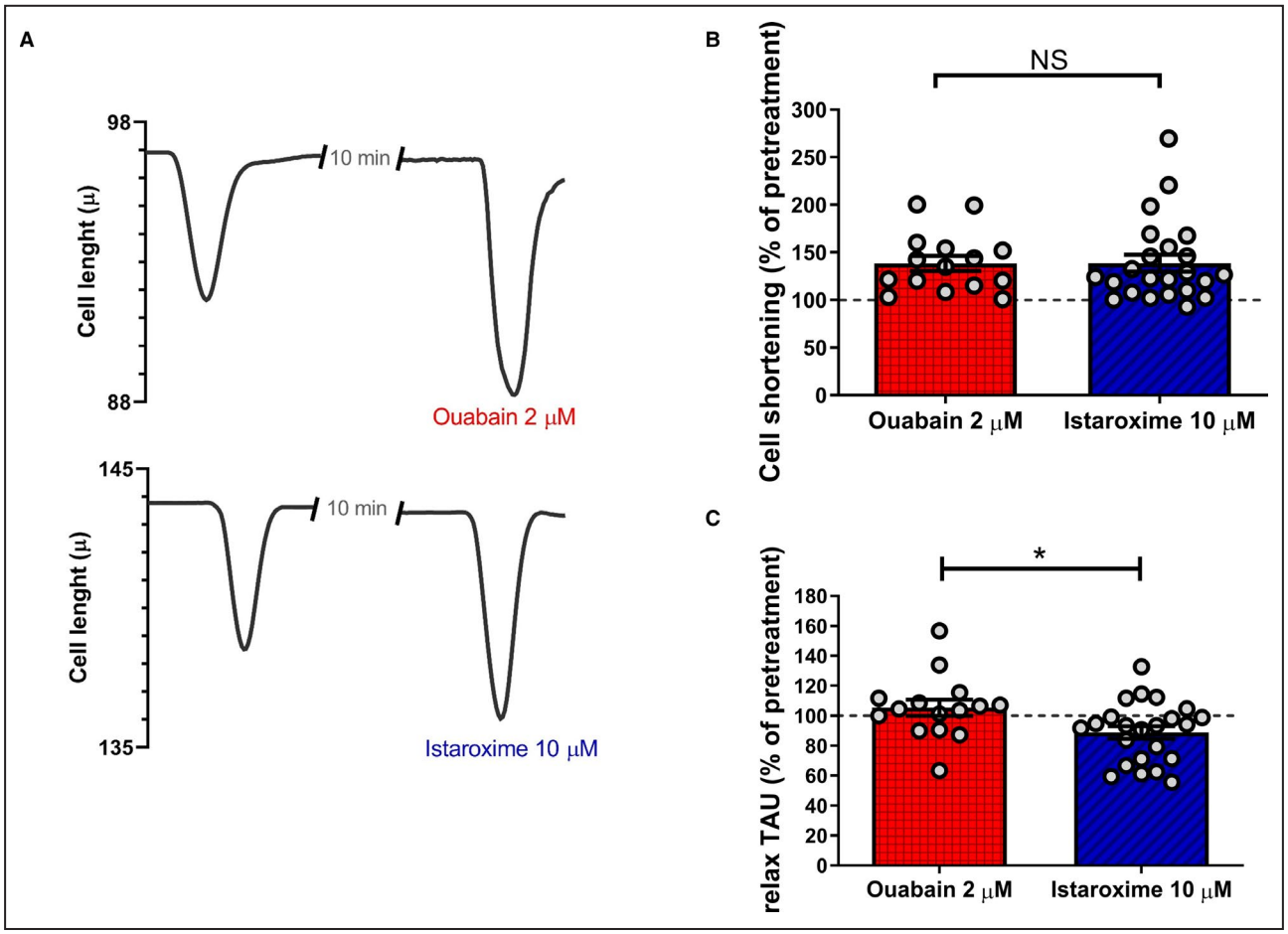

Figure 1. Two μmol/L ouabain and 10 μmol/L istaroxime are equi-inotropic but show a differential lusitropic effect. **A**, Representative traces of 1 Hz paced ventricular myocytes before and after 10 minutes of treatment with ouabain or istaroxime. **B**, Average results of cell shortening after 10 minutes of treatment expressed as percentage of pre-treatment value. **C**, Average results of relaxation TAU after 10 minutes of treatment expressed as percentage of pre-treatment value. Data presented as mean±SEM, individual points represent the data from 1 myocyte (8–10 different hearts). Unpaired Student *t* test was used to determine statistical significance; **P*<0.05.

equivalent inotropic effect measured after 10 minutes of superfusion, which is presented in Figure 1B (38.44±8.02% and 38.63±8.93%). In addition, we evaluated the percentage of change in relaxation TAU promoted by the treatment with ouabain and istaroxime. Figure 1C shows that only istaroxime promoted a reduction in shortening TAU and the lusitropic effect observed was significantly different comparing both drugs, which agreed with the fact that cells treated with ouabain showed a significantly shorter diastolic length than the cells treated with istaroxime (Figure S1).

Istaroxime Does Not Promote Cardiomyocyte Death or Activate Apoptosis

To directly compare the impact of istaroxime with that of classical NKA pharmacologic inhibition (ouabain) on direct drug-induced cardiomyocyte death, 2 approaches were followed. First, morphologic assessment of cardiomyocyte viability was measured after 24-hour culture as an index of general cell death promoted by the compared treatments. Second, to investigate the degree of activation of the apoptotic cascade, immunoblot of the pro- and antiapoptotic proteins Bax and Bcl-2 was performed in lysates from freshly isolated cardiomyocytes collected after 1-hour incubation in the absence or presence of istaroxime or ouabain at the equi-inotropic concentrations previously determined.

Figure 2A shows representative images and average data of cardiomyocyte viability taken after 24 hours of culture in which a significant reduction of cell viability was observed only when culturing cells in the presence of 2 μmol/L ouabain, while nonsignificant reduction in average cell viability was promoted by 10 μmol/L of istaroxime. Importantly, cardiomyocyte viability of the istaroxime-treated group was significantly higher than that observed with ouabain and did not significantly change with lower or higher concentrations of istaroxime (Figure S2).

In Figure 2B, representative inmunoblots and average data show how the apoptotic index, Bax/Bcl-2, is

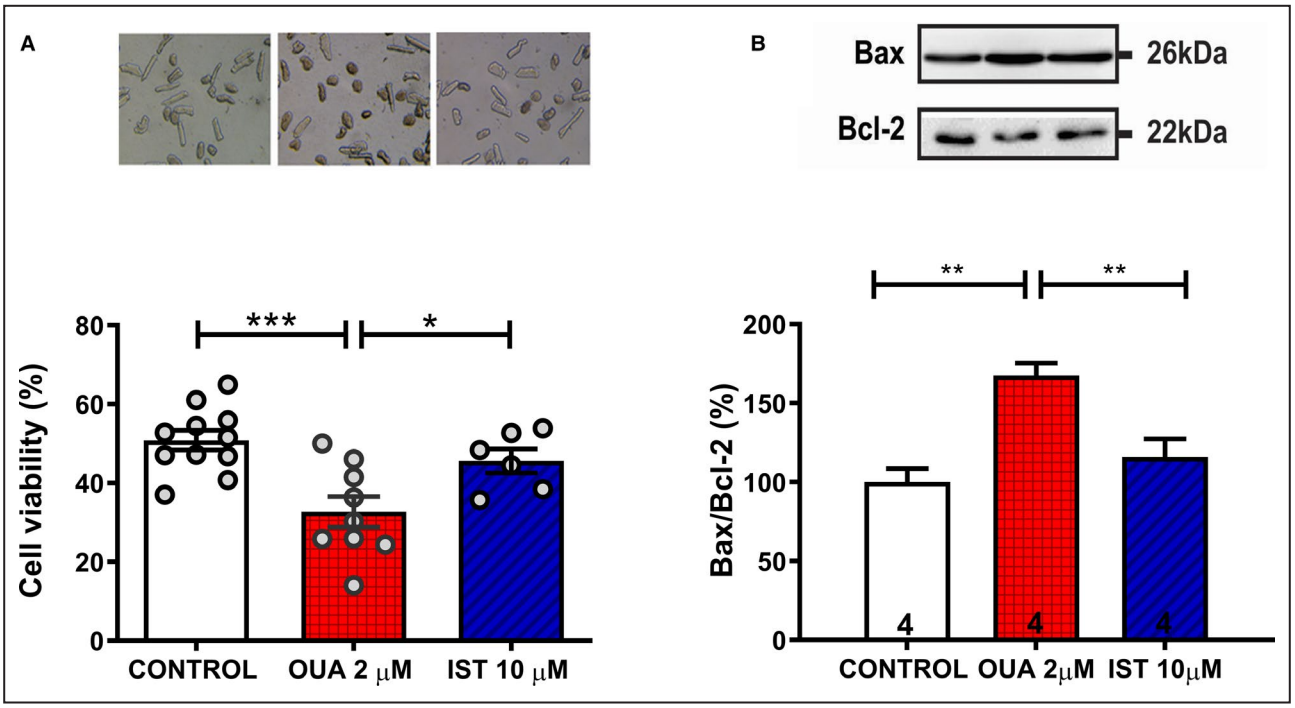

Figure 2. Differential impact of ouabain and istaroxime on cardiomyocyte viability and activation of cardiomyocyte apoptosis.
A, Primary cardiomyocytes culture was maintained for 24 hours and morphometric analysis used as an index of cardiomyocytes viability. **B**, Immunoblotting was performed to study the ratio between proapoptotic protein Bax and antiapoptotic protein Bcl-2. Data presented as mean±SEM; individual points represent the data from 1 heart. One-way ANOVA with post hoc Tukey's test was used to determine statistical significance; **P*<0.05, ***P*<0.01, and ****P*<0.001.

significantly increased by treating cardiomyocytes with ouabain and not with istaroxime.

Istaroxime Fails to Activate CaMKII

To establish the capacity of istaroxime to activate CaMKII and promote the phosphorylation of its targets, immunodetection was performed using a site-specific antibody to measure the autophosphorylation of this kinase at Thr287, which is known to relieve the inhibitory effect of the regulatory domain, resulting in kinase activation.[19] In parallel, given that an increase in reactive oxygen species production and oxidative CaMKII activation

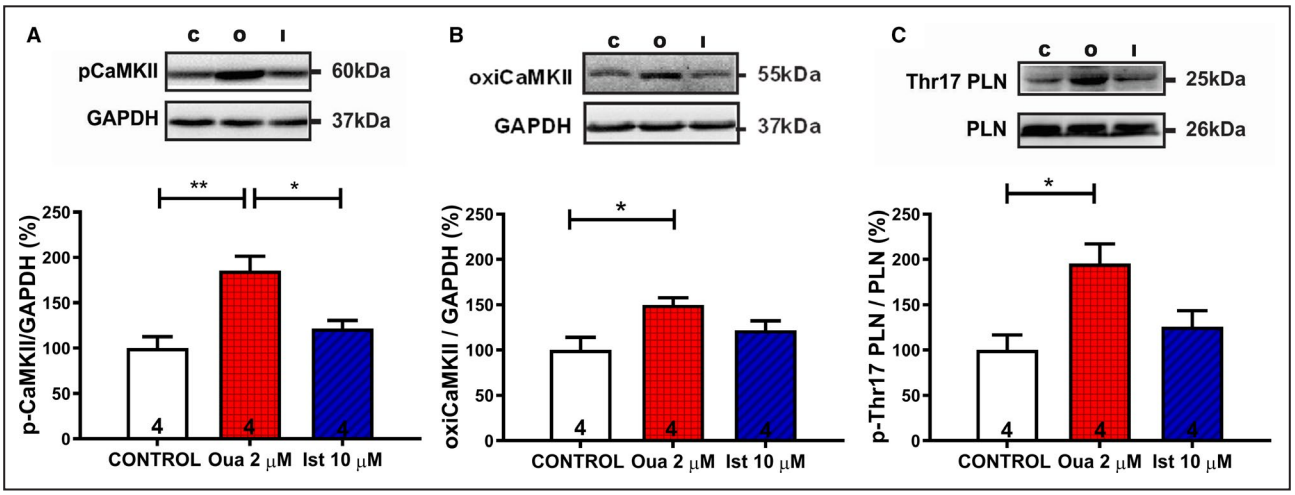

Figure 3. Ouabain activates CaMKII but istaroxime does not.
A, Representative western blot and averaged data for phospho-Thr287 CaMKII (p-CaMKII), **B**, oxidation of CaMKII (ox-CaMKII) at M281/282 and **C**, phosphorylation of phospholamban at Thr17. Data presented as mean±SEM, individual points represent the data from 1 heart. One-way ANOVA with post hoc Tukey's test was used to determine statistical significance; **P*<0.05, ***P*<0.01.

has been reported to be promoted by pharmacologic NKA inhibition,[20] we measured the activation of CaMKII caused by oxidation at M281/282. Figure 3A and 3B shows that cardiomyocytes incubation with 2 μmol/L of ouabain significantly increases both phospho-Thr286-CaMKII and oxidation of CaMKII while 10 μmol/L of istaroxime does not. Consistently, Figure 3C shows how the same differential effect was obtained when studying the CaMKII-specific substrate Thr17 of phospholamban supporting that, in contrast to ouabain, CaMKII activity is not significantly increased by therapeutic concentrations of istaroxime.

Istaroxime Does Not Increase Either Ca^{2+} Spark or Ca^{2+} Wave Frequency

Given the relevance of NKA blockade-induced Ca^{2+} overload and spontaneous Ca^{2+} release in both cell death and arrhythmogenesis,[8,9,20] we decided to compare the impact exerted by 1 hour of incubation with equi-inotropic concentrations of ouabain and istaroxime on diastolic Ca^{2+} release in the form of Ca^{2+} sparks and waves. Figure 4 depicts representative 2-dimensional Fluo-4 plots and average spark and wave frequencies, showing that only ouabain-treated cells have a significantly higher Ca^{2+} spark and wave frequency compared with control cells, indicating that istaroxime at this inotropic concentration does not promote a significant increase on diastolic, local (Ca^{2+} sparks), or propagated (Ca^{2+} waves) SR Ca^{2+} release.

Given that the dissociation of SERCA from phospholamban resulting in enhanced SR Ca^{2+} uptake has been proposed to explain the low arrhythmogenicity of istaroxime and that enhanced SR Ca^{2+} uptake has also been demonstrated to break Ca^{2+} waves into mini-waves (ie, to promote the abortion of wave propagation), which are

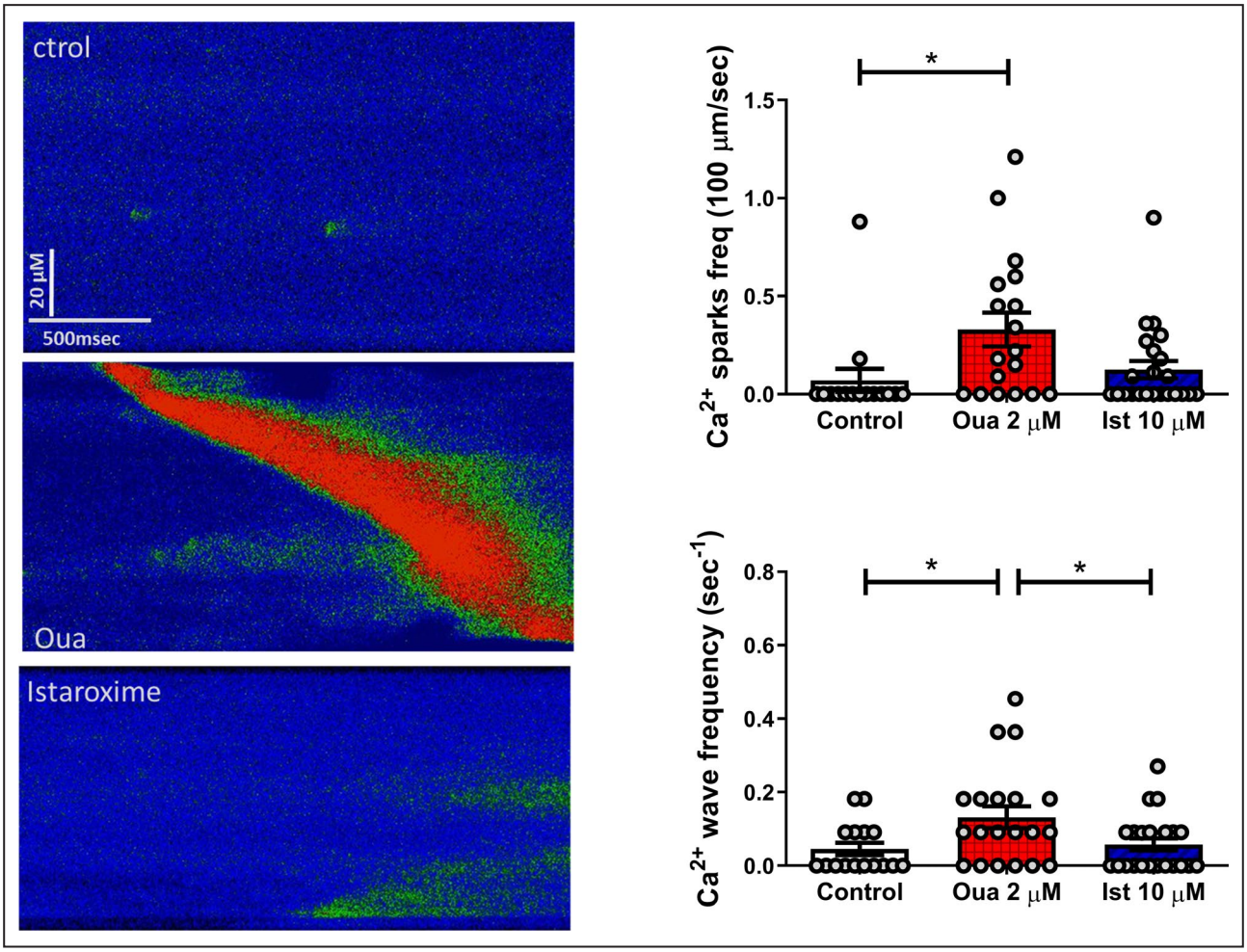

Figure 4. Istaroxime does not significantly increase Ca^{2+} spark and wave frequency.
Representative 2-dimensional Fluo-4 plots showing cytosolic Ca^{2+} signal in control and ouabain- and istaroxime-treated ventricular myocytes. Data presented as mean±SEM; individual points represent the data from 1 myocyte (from 6 hearts). One-way ANOVA with post hoc Tukey's test was used to determine statistical significance; *P<0.05.

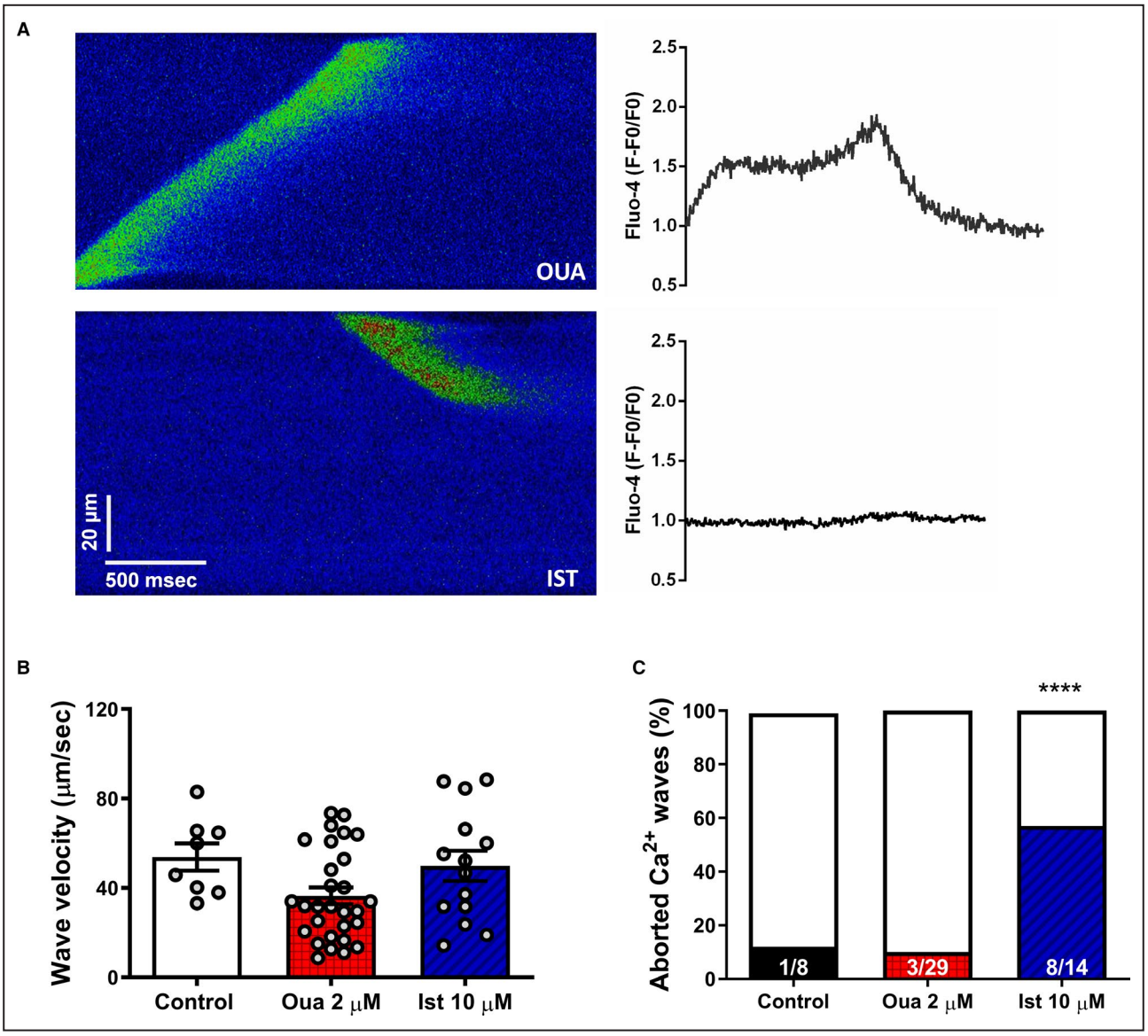

Figure 5. Propagation of Ca^{2+} waves.
A, Representative 2-dimensional (space-time) and 1-dimensional whole cell temporal profile of Fluo-4 emitted signal. **B**, Ca^{2+} waves propagation velocity; and **C,** percentage of aborted Ca^{2+} waves (mini-waves) observed in absence or presence of ouabain and istaroxime is shown. Data presented as mean±SEM, individual points represent the data from 1 myocyte (from 6 hearts). ANOVA with post hoc Tukey's test was performed to compare propagation velocity and Fisher's exact test was used to determine the statistical significance between groups when comparing incidence of mini-waves. ****P<0.0001.

less arrhythmogenic,[21–23] we paid special attention to the proportion of aborted Ca^{2+} waves found in cardiomyocytes treated with or without ouabain and istaroxime.

The whole cell intensity versus time plotting, depicted on the right of Figure 5A, clearly shows the different amount of cytosolic Ca^{2+} signal rise occurring during a whole propagated Ca^{2+} wave in the ouabain-treated myocyte, versus the much less apparent change in cytosolic Ca^{2+} promoted by a mini-wave in the istaroxime-treated cell. Interestingly, as can be seen in Figure 5C, cells incubated with istaroxime showed a significantly higher proportion of aborted Ca^{2+} waves (mini-waves), which was not associated with significant changes in propagation velocity (Figure 5B).

The Low or Absent Arrhythmogenic Capacity of Istaroxime Does Not Rely Only on SERCA/Phospholamban Dissociation

To further understand the mechanisms underlying the low arrhythmogenic profile of istaroxime, we performed experiments using phospholamban-knockout mice. Figure 6 shows representative 2-dimensional

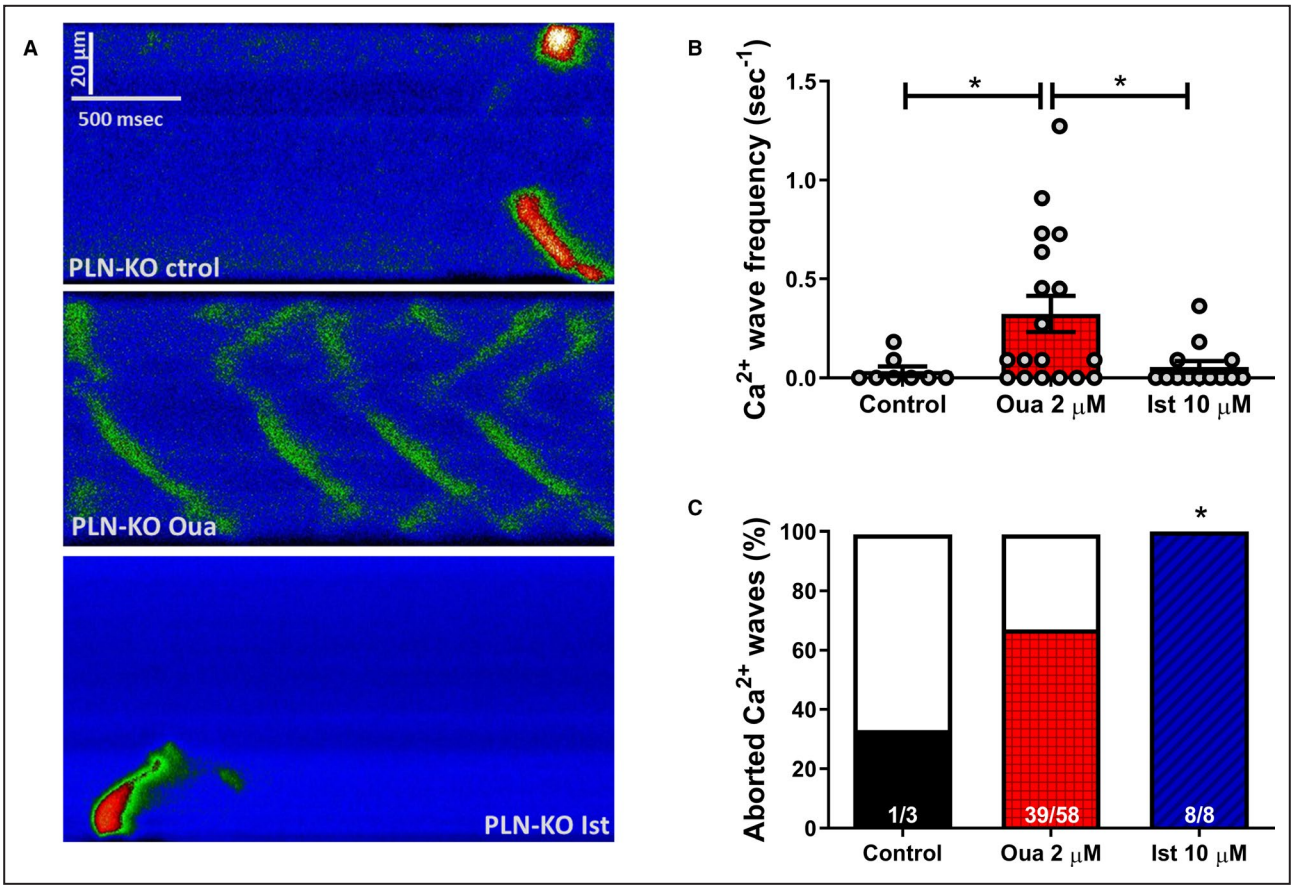

Figure 6. The differential arrhythmogenicity between ouabain and istaroxime remains present in phospholamban-knockout (PLN-KO) cardiomyocytes.

A, Representative images and **B**, average results showing Ca^{2+} wave (whole cell or mini-wave) incidence in control and ouabain- or istaroxime-treated cells. **C**, Aborted Ca^{2+} waves as percentage of total waves incidence. Data presented as mean±SEM, individual points represent the data from 1 myocyte (from 5 hearts). In **B**, ANOVA with Tukey's post hoc test was used; in **C**, indicates significance vs control calculated with Fisher's exact test using absolute values. *P<0.05.

plots of cytosolic Ca^{2+} recorded in cardiomyocytes lacking phospholamban expression, after 1-hour incubation in absence or presence of 2 µmol/L of ouabain or 10 µmol/L of istaroxime.

Consistent with the previously discussed concept regarding Ca^{2+} uptake acceleration and Ca^{2+} wave abortion, phospholamban-knockout myocytes showed a high percentage of aborted Ca^{2+} waves. Nevertheless, as shown in Figure 6C, this percentage was higher in istaroxime-treated cells (100%) than in ouabain-treated cells (67%).

More importantly for the aim of the experiment, average results shown in Figure 6B demonstrate that a significant difference on Ca^{2+} wave frequency between ouabain- and istaroxime-treated cells remains present even if phospholamban is absent, supporting that in addition to SERCA/phospholamban dissociation, another mechanism underlies the different impact that both inotropes have on spontaneous SR Ca^{2+} release.

DISCUSSION

Major findings of this study are:

- Istaroxime can increase cardiomyocyte inotropy without significantly promoting CaMKII activity or direct cardiomyocyte toxicity.
- Regarding the mechanisms underlying the lower risk of cardiac arrhythmia observed when istaroxime was compared with classical NKA inhibitors in both preclinical and clinical studies,[5,7,13,24] we provide here new and alternative explanations. We show that istaroxime breaks arrhythmogenic Ca^{2+} waves into mini-waves, which are recognized as less arrhythmogenic. Finally, this study demonstrates that in addition to SERCA/phospholamban dissociation, other mechanisms such as the lack of CaMKII activation underlie the low arrhythmogenic profile of istaroxime.

Role of CaMKII Activation and Ca^{2+} Mishandling on Cardiomyocyte Death

It has been established that CaMKII activation mediates different forms of cardiomyocyte death, including apoptosis and necroptosis promoted by several myocardial injuries such as ischemia-reperfusion or doxorubicin exposure.[25,26] Between other effects, CaMKII can trigger the opening of a mitochondrial permeability transition pore,[26] constituting a nodal point between the mechanisms that lead to cardiomyocyte death.

We have previously shown that digitalis-induced cardiotoxicity involves CaMKII-dependent phosphorylation of the cardiac ryanodine receptors,[8,16] leading to increased diastolic SR Ca^{2+} release, which is visualized as an increased number of sparks and waves. In particular, we previously showed that both the inhibition of cardiac isoform of ryanodine receptors mediated spontaneous SR Ca^{2+} release and of the mitochondrial Ca^{2+} uniporter can prevent ouabain-induced cardiomyocyte death,[16] suggesting that increased SR Ca^{2+} release, and Ca^{2+} transference to mitochondria, plays a role in ouabain-induced cardiomyocyte death. Based on this concept, herein we compared the capacity of ouabain and istaroxime to promote CaMKII activation and to increase Ca^{2+} spark and wave frequency, and we observed that, in fact, all these events were enhanced by ouabain but not by istaroxime. The lack of istaroxime-induced spark and wave occurrence can be linked to both low cardiomyocyte death and to the lower arrhythmogenicity of this compound. However, an acceleration of SR Ca^{2+} uptake, like the one promoted by istaroxime, has also been reported to promote rather than to prevent cardiomyocyte death in other models.[22,27] Thereby, the capacity to activate CaMKII exerted by classical NKA inhibitors but not istaroxime, emerges as a complementary benefit and seems to shed light into the improved inotropic/cardiotoxic balance of istaroxime.

Given that one of the aims of this study is to identify the cardiotoxic potential of istaroxime by promoting loss of fully differentiated ventricular cardiomyocytes, we did not explore in depth the proportional contribution of necrosis, necroptosis, or apoptosis to cardiomyocyte death in the context of inotropic treatment. However, it is worth establishing whether or not istaroxime leads to cardiomyocyte death at inotropic concentrations. Additionally, if we consider that a frequent limitation of anticancer therapies is the risk of cardiotoxicity, it is relevant to note that the concentration of istaroxime used here was higher than the one reported as efficient to promote tumoral cell death,[10] suggesting that antitumoral effect can be reached without promoting cardiotoxicity.

An additional finding of this work is that oxidation of CaMKII was promoted by ouabain but not by istaroxime. It has been shown that digitalis, by directly interacting with NKA, can activate signaling pathways in parallel to the changes in Na^+ and Ca^{2+} balance these drugs promote, and it involves the production of reactive oxygen species that oxidate CaMKII and other targets.[20] This suggest that istaroxime could be less potent or even fail to trigger this signaling pathway and will probably motivate future study.

Insights on the Low Arrhythmogenic Profile of Istaroxime

It is widely accepted that NKA inhibition can promote ventricular arrhythmias attributable to spontaneous SR Ca^{2+} release, mediating Ca^{2+}-induced depolarizing currents such as Na^+/Ca^{2+} exchanger-mediated current, which finally leads to delayed after-depolarizations,[28,29] which, if they reach the threshold voltage, will initiate action potentials and ectopic beats.

Considering our previous results showing that CaMKII activation mediates digitalis-induced arrhythmias and that we found here that istaroxime does not activate CaMKII, we propose that this can explain, at least in part, the differential effect of ouabain and istaroxime on Ca^{2+} spark/wave frequency and arrhythmogenesis.

At present, the proposed mechanism to explain istaroxime's low arrhythmogenic profile has been its capacity to boost SR Ca^{2+} uptake attributable to SERCA-phospholamban dissociation. However, whether the latter can explain a low frequency of SR Ca^{2+} waves is not apparent to us. In this regard, an important novel finding of this study, which can be explained by an istaroxime-dependent acceleration of Ca^{2+} uptake, is the high proportion of mini-waves we found in istaroxime-treated cardiomyocytes. It has been previously shown that relieving SERCA from phospholamban can break Ca^{2+} waves propagation, and this aborted Ca^{2+} waves or mini-waves have a lower potential to induce delayed after-depolarizations than fully propagated Ca^{2+} waves given that less Ca^{2+} is released from SR to cytosol and the subsequent Na^+/Ca^{2+} exchanger-mediated current is less likely to reach the threshold voltage and generate ectopic beats.[21,22]

We propose that both the acceleration of Ca^{2+} uptake promoted by istaroxime and the lack of CaMKII activation in cardiomyocytes treated with istaroxime are the basis of its low arrhythmogenic profile, and this is supported by the results we show here in phospholamban-knockout myocytes in which, even in absence of phospholamban, the cells treated with istaroxime show a lower Ca^{2+} wave frequency than ouabain-treated cells. Furthermore, a lower CaMKII activity could explain the higher proportion of mini-waves

we found in phospholamban-knockout myocytes treated with istaroxime. In addition to the role of Ca^{2+} uptake, it is recognized that cardiac isoform of ryanodine receptors sensitivity affects Ca^{2+} wave propagation,[30,31] and this sensitivity is known to be increased by CaMKII-dependent phosphorylation.[32]

Overall, our results support that the lack of CaMKII activation is an additional mechanism underlying the low arrhythmogenicity of istaroxime.

CONCLUSIONS

In summary, we unveil new mechanisms that underlie the low cardiotoxic profile of istaroxime. In particular, we show that, different from other NKA inhibitors, istaroxime neither activates CaMKII nor increases spark and wave frequency nor promotes cardiomyocyte death. In addition, our results show that istaroxime breaks Ca^{2+} wave propagation, leading to fewer arrhythmogenic mini-waves.

Overall, the evidence presented here strongly supports the safety of this compound for being used at clinically relevant concentrations that are accepted to improve heart function and to act as an antitumoral drug.

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Disclosures

None.

Supplementary Material

Figures S1–S2

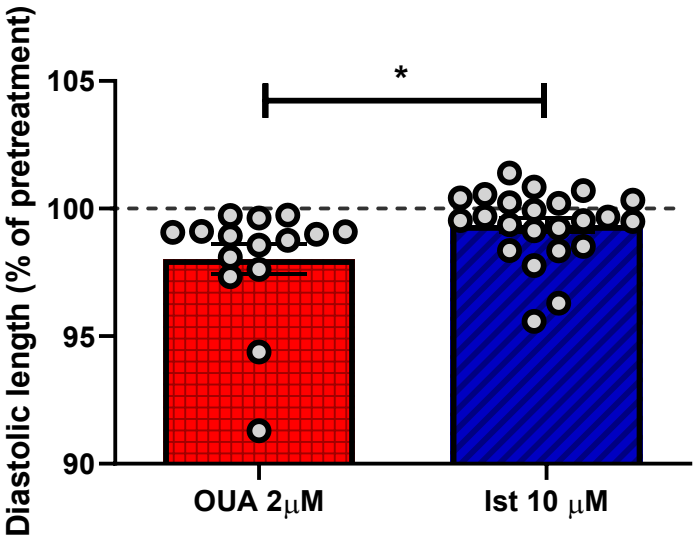
REFERENCES

1. Maack C, Eschenhagen T, Hamdani N, Heinzel FR, Lyon AR, Manstein DJ, Metzger J, Papp Z, Tocchetti CG, Yilmaz MB, et al. Treatments targeting inotropy. *Eur Heart J*. 2019;40:3626–3644. DOI: 10.1093/eurheartj/ehy600.
2. Digitalis IG. The effect of digoxin on mortality and morbidity in patients with heart failure. *N Engl J Med*. 1997;336:525–533. DOI: 10.1056/NEJM199702203360801.
3. Ferrari F, Santander I, Stein R. Digoxin in atrial fibrillation: an old topic revisited. *Curr Cardiol Rev*. 2020;16:141–146. DOI: 10.2174/1573403X15666190618110941.
4. Bavendiek U, Berliner D, Dávila LA, Schwab J, Maier L, Philipp SA, Rieth A, Westenfeld R, Piorkowski C, Weber K, et al. Rationale and design of the DIGIT-HF trial (digitoxin to improve outcomes in patients with advanced chronic heart failure): a randomized, double-blind, placebo-controlled study. *Eur J Heart Fail*. 2019;21:676–684. DOI: 10.1002/ejhf.1452.
5. Carubelli V, Zhang Y, Metra M, Lombardi C, Felker GM, Filippatos G, O'Connor CM, Teerlink JR, Simmons P, Segal R, et al. Treatment with 24 hour istaroxime infusion in patients hospitalised for acute heart failure: a randomised, placebo-controlled trial. *Eur J Heart Fail*. 2020;22:1684–1693. DOI: 10.1002/ejhf.1743.
6. Shah SJ, Blair JEA, Filippatos GS, Macarie C, Ruzyllo W, Korewicki J, Bubenek-Turconi SI, Ceracchi M, Bianchetti M, Carminati P, et al. Effects of istaroxime on diastolic stiffness in acute heart failure syndromes: results from the hemodynamic, echocardiographic, and neurohormonal effects of istaroxime, a novel intravenous inotropic and lusitropic agent: a randomized controlled trial in patients hospitalized with heart failure (HORIZON-HF) trial. *Am Heart J*. 2009;157:1035–1041. DOI: 10.1016/j.ahj.2009.03.007.
7. Alemanni M, Rocchetti M, Re D, Zaza A. Role and mechanism of subcellular Ca^{2+} distribution in the action of two inotropic agents with different toxicity. *J Mol Cell Cardiol*. 2011;50:910–918. DOI: 10.1016/j.yjmcc.2011.02.008.
8. Sapia L, Palomeque J, Mattiazzi A, Petroff MV. Na+/K+-ATPase inhibition by ouabain induces CAMKII-dependent apoptosis in adult rat cardiac myocytes. *J Mol Cell Cardiol*. 2010;49:459–468. DOI: 10.1016/j.yjmcc.2010.04.013.
9. Gonano LA, Sepulveda M, Rico Y, Kaetzel M, Valverde CA, Dedman J, Mattiazzi A, Vila PM. Calcium-calmodulin kinase II mediates digitalis-induced arrhythmias. *Circ Arrhythm Electrophysiol*. 2011;4:947–957. DOI: 10.1161/CIRCEP.111.964908.
10. Alevizopoulos K, Dimas K, Papadopoulou N, Schmidt EM, Tsapara A, Alkahtani S, Honisch S, Prousis KC, Alarifi S, Calogeropoulou T, et al. Functional characterization and anti-cancer action of the clinical phase II cardiac Na+/K+ ATPase inhibitor istaroxime: in vitro and in vivo properties and cross talk with the membrane androgen receptor. *Oncotarget*. 2016;7:24415–24428. DOI: 10.18632/oncotarget.8329.
11. Mijatovic T, Van Quaquebeke E, Delest B, Debeir O, Darro F, Kiss R. Cardiotonic steroids on the road to anti-cancer therapy. *Biochim Biophys Acta*. 2007;1776:32–57. DOI: 10.1016/j.bbcan.2007.06.002.
12. Triana-Martínez F, Picallos-Rabina P, Da Silva-Álvarez S, Pietrocola F, Llanos S, Rodilla V, Soprano E, Pedrosa P, Ferreirós A, Barradas M, et al. Identification and characterization of cardiac glycosides as senolytic compounds. *Nat Commun*. 2019;10:4731. DOI: 10.1038/s41467-019-12888-x.
13. Ferrandi M, Barassi P, Tadini-Buoninsegni F, Bartolommei G, Molinari I, Tripodi MG, Reina C, Moncelli MR, Bianchi G, Ferrari P. Istaroxime stimulates SERCA2a and accelerates calcium cycling in heart failure by relieving phospholamban inhibition. *Br J Pharmacol*. 2013;169:1849–1861. DOI: 10.1111/bph.12278.
14. Liu B, Lou Q, Smith H, Velez-Cortes F, Dillmann WH, Knollmann BC, Armoundas AA, Gyorke S. Conditional up-regulation of SERCA2a exacerbates RyR2-dependent ventricular and atrial arrhythmias. *Int J Mol Sci*. 2020;21:2535. DOI: 10.3390/ijms21072535.
15. Vila-Petroff M, Salas MA, Said M, Valverde CA, Sapia L, Portiansky E, Hajjar RJ, Kranias EG, Mundina-Weilenmann C, Mattiazzi A. CAMKII inhibition protects against necrosis and apoptosis in irreversible ischemia-reperfusion injury. *Cardiovasc Res*. 2007;73:689–698. DOI: 10.1016/j.cardiores.2006.12.003.
16. Gonano LA, Sepulveda M, Morell M, Toteff T, Racioppi MF, Lascano E, Negroni J, Fernandez Ruocco MJ, Medei E, Neiman G, et al. Non-beta-blocking carvedilol analog, VK-II-86, prevents ouabain-induced cardiotoxicity. *Circ J*. 2018;83:41–51. DOI: 10.1253/circj.CJ-18-0247.
17. Picht E, Zima AV, Blatter LA, Bers DM. Sparkmaster: automated calcium spark analysis with ImageJ. *Am J Physiol Cell Physiol*. 2007;293:C1073–C1081. DOI: 10.1152/ajpcell.00586.2006.
18. Sepulveda M, Gonano LA, Back TG, Chen SR, Vila PM. Role of CAMKII and ROS in rapid pacing-induced apoptosis. *J Mol Cell Cardiol*. 2013;63:135–145. DOI: 10.1016/j.yjmcc.2013.07.013.
19. Erickson JR, He BJ, Grumbach IM, Anderson ME. CAMKII in the cardiovascular system: sensing redox states. *Physiol Rev*. 2011;91:889–915. DOI: 10.1152/physrev.00018.2010.
20. Ho H-T, Liu B, Snyder JS, Lou Q, Brundage EA, Velez-Cortes F, Wang H, Ziolo MT, Anderson ME, Sen CK, et al. Ryanodine receptor

phosphorylation by oxidized CamKII contributes to the cardiotoxic effects of cardiac glycosides. *Cardiovasc Res*. 2014;101:165–174. DOI: 10.1093/cvr/cvt233.
21. Bai Y, Jones PP, Guo J, Zhong X, Clark RB, Zhou Q, Wang R, Vallmitjana A, Benitez R, Hove-Madsen L, et al. Phospholamban knockout breaks arrhythmogenic Ca(2)(+) waves and suppresses catecholaminergic polymorphic ventricular tachycardia in mice. *Circ Res*. 2013;113:517–526. DOI: 10.1161/CIRCRESAHA.113.301678.
22. Valverde CA, Mazzocchi G, Di Carlo MN, Ciocci Pardo A, Salas N, Ragone MI, Felice JI, Cely-Ortiz A, Consolini AE, Portiansky E, et al. Ablation of phospholamban rescues reperfusion arrhythmias but exacerbates myocardium infarction in hearts with Ca^{2+}/calmodulin kinase II constitutive phosphorylation of ryanodine receptors. *Cardiovasc Res*. 2019;115:556–569. DOI: 10.1093/cvr/cvy213.
23. Huser J, Bers DM, Blatter LA. Subcellular properties of [Ca^{2+}]i transients in phospholamban-deficient mouse ventricular cells. *Am J Physiol*. 1998;274:H1800–H1811. DOI: 10.1152/ajpheart.1998.274.5.H1800.
24. Bossu A, Kostense A, Beekman HDM, Houtman MJC, van der Heyden MAG, Vos MA. Istaroxime, a positive inotropic agent devoid of proarrhythmic properties in sensitive chronic atrioventricular block dogs. *Pharmacol Res*. 2018;133:132–140. DOI: 10.1016/j.phrs.2018.05.001.
25. Tscheschner H, Meinhardt E, Schlegel P, Jungmann A, Lehmann LH, Muller OJ, Most P, Katus HA, Raake PW. CAMKII activation participates in doxorubicin cardiotoxicity and is attenuated by moderate GRP78 overexpression. *PLoS One*. 2019;14:e0215992. DOI: 10.1371/journal.pone.0215992.
26. Zhang T, Zhang Y, Cui M, Jin LI, Wang Y, Lv F, Liu Y, Zheng W, Shang H, Zhang J, et al. CAMKII is a RIP3 substrate mediating ischemia- and oxidative stress-induced myocardial necroptosis. *Nat Med*. 2016;22:175–182. DOI: 10.1038/nm.4017.
27. Zhang T, Guo T, Mishra S, Dalton ND, Kranias EG, Peterson KL, Bers DM, Brown JH. Phospholamban ablation rescues sarcoplasmic reticulum Ca(2+) handling but exacerbates cardiac dysfunction in CAMKIIdelta(C) transgenic mice. *Circ Res*. 2010;106:354–362. DOI: 10.1161/CIRCRESAHA.109.207423.
28. Xie LH, Weiss JN. Arrhythmogenic consequences of intracellular calcium waves. *Am J Physiol Heart Circ Physiol*. 2009;297:H997–H1002. DOI: 10.1152/ajpheart.00390.2009.
29. Schlotthauer K, Bers DM. Sarcoplasmic reticulum Ca(2+) release causes myocyte depolarization. Underlying mechanism and threshold for triggered action potentials. *Circ Res*. 2000;87:774–780. DOI: 10.1161/01.res.87.9.774.
30. Ullrich ND, Valdivia HH, Niggli E. PKA phosphorylation of cardiac ryanodine receptor modulates SR luminal Ca^{2+} sensitivity. *J Mol Cell Cardiol*. 2012;53:33–42. DOI: 10.1016/j.yjmcc.2012.03.015.
31. Swietach P, Spitzer KW, Vaughan-Jones RD. Modeling calcium waves in cardiac myocytes: importance of calcium diffusion. *Front Biosci (Landmark Ed)*. 2010;15:661–680. DOI: 10.2741/3639.
32. Niggli E, Ullrich ND, Gutierrez D, Kyrychenko S, Polakova E, Shirokova N. Posttranslational modifications of cardiac ryanodine receptors: Ca(2+) signaling and EC-coupling. *Biochim Biophys Acta*. 2013;1833:866–875. DOI: 10.1016/j.bbamcr.2012.08.016.

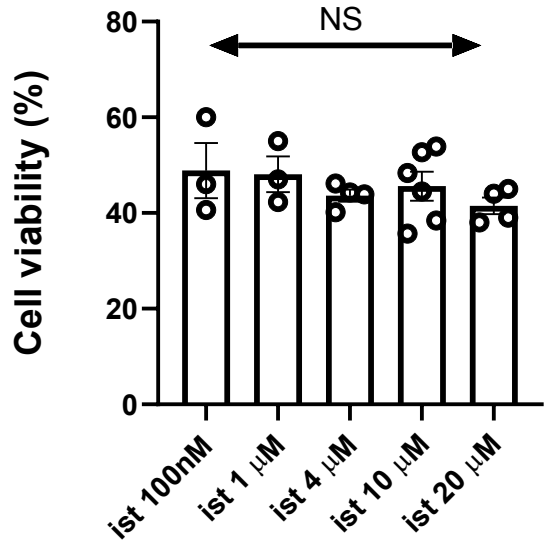
Supplemental Material

Figure S1. Percentual diastolic length change after 10 minutes of treatment is significantly different between ouabain and istaroxime treated cells.



Data presented as mean±SEM, individual points represent the data from 1 myocyte (8-10 different hearts). Unpaired student's t-test was used to determine statistical significance, *= p< 0.05.

Figure S2. Cardiomyocytes viability after 24 hours in culture with different concentrations of istaroxime.



Data presented as mean±SEM, individual points represent the data from 1 heart. ANOVA with Tukey's Post Hoc test was used, and not significant differences were found when comparing differences between each individual group.