Cardiac Glycosides as Novel Inhibitors of HERG Channel Trafficking

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Running title: Cardiac Glycosides and hERG trafficking

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Number of text pages: 35

Number of tables: 1

Number of figures: 8

Number of references: 38

Number of words in the Abstract: 246

Number of words in the Introduction: 565

Number of words in the Discussion: 1475

Abbreviations: acLQTS, acquired long QT syndrome, APD, action potential duration, AV, atrioventricular, ECG, electrocardiogram, ER, endoplasmic reticulum, HA, hemagglutinin, HEK, human embryonic kidney, hERG, human ether a-go-go-related gene, I_{Kr}, rapidly activating delayed rectifier K current, I_{Ks}, slowly activating delayed rectifier K current, LQTS, long QT syndrome, SR, sarcoplasmic reticulum, TdP, torsades de pointes

Section assignment: Cardiovascular

Abstract

Direct block of the cardiac potassium channel hERG by a large, structurally diverse group of therapeutic compounds causes drug-induced QT prolongation and torsades de pointes arrhythmias. In addition, several therapeutic compounds have been identified more recently that prolong the QT interval by inhibition of hERG trafficking to the cell surface. We used a surface expression assay to identify novel compounds that interfere with hERG trafficking and found that cardiac glycosides are potent inhibitors of hERG expression at the cell surface. Further investigation of digitoxin, ouabain and digoxin revealed that all three cardiac glycosides reduced expression of the fully-glycosylated cell surface form of hERG on Western blots indicating that channel exit from the endoplasmic reticulum is blocked. Similarly, hERG currents were reduced with nanomolar affinity on long-term exposure. HERG trafficking inhibition was initiated by cardiac glycosides through direct block of Na/K pumps and not via off-target interactions with hERG or another closely associated protein in its processing or export pathway. In isolated guinea pig myocytes long-term exposure to 30 nM of the clinically used drugs, digoxin or digitoxin, reduced hERG/I_{Kr} currents by about 50% while three other cardiac membrane currents, I_{K1}, I_{Ks} and I_{Ca} were not affected. Importantly, 100 nM digitoxin prolonged action potential duration on long-term exposure consistent with a reduction in hERG/I_{Kr} channel number. Thus, cardiac glycosides are able to delay cardiac repolarization at nanomolar concentrations via hERG trafficking inhibition and this may contribute to the complex electrocardiographic changes seen with compounds such as digitoxin.

Introduction

The cardiac potassium channel hERG encodes the α-subunit of the rapid delayed rectifier current I_{Kr} in the heart which contributes prominently to terminal (phase3) repolarization in human ventricular myocytes. The strategic position occupied by hERG/I_{Kr} channels in cardiac repolarization was uncovered first in patients with familial long QT syndrome (LQTS2) with the identification of inherited mutations in the hERG gene (KCNH2) that were ultimately shown to reduce hERG/I_{Kr} currents (Sanguinetti et al. 1995; Sanguinetti and Tristani-Firouzi, 2006). In symptomatic patients, LQTS is characterized by a prolongation of the QT interval on the electrocardiogram which is considered a surrogate marker for ventricular repolarization at the cellular level and, as a direct consequence, an increased propensity to develop arrhythmias that may deteriorate into ventricular fibrillation and cause sudden cardiac arrest (Keating and Sanguinetti, 2001). Similar electrocardiographic abnormalities can be induced via direct block of hERG/I_{Kr} channels by a large group of structurally diverse therapeutic compounds including many antiarrhythmics, antihistamines, antipsychotics and antibiotics (Fermini and Fossa, 2003), a phenomenon known as drug-induced or acquired long QT syndrome (acLQTS). Since acLQTS due to unintended block of hERG channels is largely preventable, regulatory agencies have removed certain drugs such as terfenedine and cisapride from the market place and restricted the use of others. Based on this well-recognized susceptibility of hERG for block by small organic molecules, regulatory guidelines are currently being instituted for the development of novel therapeutic compounds that include the assessment of direct hERG block in electrophysiological measurements, ion flux or binding assays (Fenichel et al., 2004).

More recently, we have described a novel mechanism for acLQTS that goes undetected in conventional safety studies. We have shown that at least two therapeutic compounds, arsenic

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trioxide, which is used in the treatment of acute promelocytic leukemia, and the anti-protozoical agent, pentamidine, reduce hERG/I_{Kr} currents not by direct block but by inhibition of hERG/I_{Kr} trafficking to the cell surface (Ficker et al. 2004; Kuryshev et al. 2005). In contrast to direct hERG block where a detailed mechanistic understanding of the 'universal' drug binding site of hERG may be exploited in the near future to avoid unintended drug-channel interactions (Sanguinetti and Tristani-Firouzi, 2006), less is known about how small organic molecules impede hERG trafficking and whether certain compound classes exist that are particularly prone to inhibit hERG trafficking. A critical first step depends on the systematic identification of novel compound classes that reduce cell surface expression of hERG with high specificity. To accomplish this goal, we have recently developed a high throughput antibody-based chemiluminescent assay to monitor heterologous expression of hERG channels at the cell surface (Wible et al., 2005).

Using this assay we have discovered that cardiac glycosides constitute a novel compound class that decreases hERG surface trafficking with nanomolar affinity. Cardiac glycosides are well-known specific inhibitors of Na^+/K^+ ATPases, the molecular pumps that maintain the steep Na^+ and K^+ ion gradients at the plasma membrane, and have been used for many years in the treatment of congestive heart failure and/or atrial fibrillation (Hauptman and Kelly, 1999; Gheorghiade et al., 2004; Wasserstrom and Aistrup, 2005). However, due to a narrow therapeutic window their clinical use has always been troubled by cardiac toxicity involving lethal arrhythmias (Ma et al., 2001; Grimard et al., 2005). The identification of cardiac glycosides as potent hERG trafficking inhibitors will further our understanding of the mechanisms that may underlie adverse cardiac events triggered by cardiac glycosides.

Methods

Cell culture. Human embryonic kidney (HEK) 293 cells stably expressing hERG WT or hERG WT HA$_{ex}$ with an extracellular hemagglutinin (HA) tag (Ficker et al, 2003; Ficker et al, 2004) were maintained at 37°C, 5% CO$_2$ in DMEM supplemented with 10% fetal bovine serum, L-glutamine, penicillin/streptomycin (DMEM complete medium) and geneticin. To assess the effects of extracellular potassium concentration on hERG trafficking, HEK/hERG WT cells were cultured overnight at 37°C, 5% CO$_2$ in a serum-free salt solution (120 mM NaCl, 1 mM CaCl$_2$, 1 mM MgSO$_4$, 0.2 mM NaH$_2$PO$_4$, 0.15 mM Na$_2$HPO$_4$, 4 mM NaHCO$_3$, 5 mM glucose, 1 mM pyruvic acid, 10 mM lactic acid, 20 mM HEPES, pH 7.4) supplemented with MEM essential amino acids (Invitrogen, 11130-051), MEM non-essential amino acids (Invitrogen, 11140-050), and MEM vitamins (Invitrogen, 11120-052) with either 5mM (5K-sf), 2mM (2K-sf) or without any KCl added (0K-sf). For transient transfections, we used HEK 293 cells, Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), pcDNA3-hERG WT and pCMV Ouabain^r vector (BD Biosciences Pharmingen, San Diego, CA) encoding a rat ouabain-resistant Na,K-ATPase α1-subunit. Human Kv1.5 (hKv1.5) was stably expressed in mouse L cells and maintained in DMEM complete medium supplemented with geneticin. Chinese hamster ovary (CHO) cells were stably transfected with pcDNA3-KCNQ1(KvLQT1)/pZEOSV2-KCNE1 (minK) and maintained in MEM-α medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin/streptomycin, and geneticin/zeocin for selection.

Chemiluminescence detection of cell surface hERG protein. Stably transfected HEK/hERGWT HA$_{ex}$ cells were plated at 40,000 cells/well in a 96-well plate. After overnight incubation with test compounds, cells were fixed, blocked and incubated for one hour with rat anti-HA-monoclonal antibody (Roche). After washing, HRP-conjugated goat anti-rat IgG

(Jackson ImmunoResearch Laboratories Inc, West Grove, PA) and the dsDNA stain SYBR

Green (Invitrogen, Carlsbad, CA) were added for one hour (Wible et al., 2005). SYBR Green

fluorescence was measured to determine cell numbers. Chemiluminescent signals were

developed using SuperSignal (Pierce) and captured in a Beckman Coulter DTX 880 plate reader

(Beckman Coulter, Fullerton, CA). Luminescence signals were corrected for cell loss as

measured by SYBR Green fluorescence with the data presented as normalized surface expression

relative to control.

Western blot analysis. Antibodies used in the present study have been described

previously (Ficker et al, 2003; Ficker et al, 2004). Briefly, HEK/hERG and L/hKv1.5 cells were

solubilized for 1h at 4°C in a lysis buffer containing 1% Triton X-100 and protease inhibitors

(Complete, Roche Diagnostics, Indianapolis, IN). Protein concentrations were determined by the

BCA method (Pierce, Rockford, IL). Proteins were separated on SDS polyacrylamide gels,

transferred to polyvinylidene difluoride membranes and developed using either rabbit anti-hERG

polyclonal antibody (HERG 519) or rabbit anti-hKv1.5 polyclonal antibody followed by ECL

Plus (GE Healthcare, Piscataway, NJ). For quantitative analysis, chemiluminescence signals

were captured directly on a Storm PhosphorImager (GE Healthcare) (Ficker et al, 2003).

Cellular electrophysiology. HEK/hERG, L/hKv1.5 and CHO/KCNQ1-KCNE1 cells

were recorded using patch pipettes filled with: 100 mM K-aspartate, 20mM KCl, 2mM $MgCl_2$,

1mM $CaCl_2$, 10mM EGTA, and 10mM HEPES (pH 7.2). The extracellular solution was: 140

mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, and 10mM glucose (pH

7.4). To study long-term drug effects on currents, cardiac glycosides were added for 16-20 hrs

(overnight) prior to recording. Stock solutions for all compounds were prepared in DMSO.

Final DMSO concentrations in drug-containing solutions did not exceed 0.1%. The cardiac

membrane currents I_{Kr}, I_{Ks} and I_{K1} were recorded in freshly isolated ventricular guinea pig myocytes to study short term effects of cardiac glycosides or in guinea pig myocytes cultured overnight in M199 medium at 37°C in the presence of 5% CO_2 to study long term effects (Ficker et al., 2003) using the following intracellular solution: 119 mM K-gluconate, 15 mM KCl, 3.75 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, 4mM K-ATP, 14 mM phosphocreatine, 0.3 mM Tris-GTP and 50U/ml creatine phosphokinase (pH 7.2). The extracellular solution was 132 mM NaCl, 4 mM KCl, 1.2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES and 5mM glucose (pH 7.4). In recordings of cardiac potassium currents Ca^{2+} currents were blocked with 1µM nisoldipine. The specific hERG/I_{Kr} blocker E4031 was used to isolate I_{Kr} currents. Cardiac calcium currents were recorded using the following extracellular solution: 137 mM NaCl, 5.4 mM CsCl, 1.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4). The intracellular solution was 130 mM $CsMeSO_4$, 20 mM TEACl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, 14 mM Tris-phosphocreatine, 0.3 mM Tris-GTP, and 50U/ml creatine phosphokinase (pH 7.2). Action potentials were recorded either in freshly isolated ventricular guinea pig myocytes or in myocytes kept in short-term (overnight) culture using the perforated patch technique to preserve the intracellular milieu of the intact cardiomyocyte. Briefly, patch pipettes were back-filled with: 120 mM K-aspartate, 20 mM KCl, 10 mM NaCl, 2mM $MgCl_2$, 5 mM HEPES (pH 7.3) supplemented with 240 µg/ml amphotericin-B (Sigma, St. Louis, MO). The extracellular solution was Tyrode's solution (137 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose and 10 mM HEPES, pH 7.3). Following seal formation access resistance was continuously monitored. Experimental protocols were initiated after the access resistance dropped below 40MΩ. Action potentials were elicited in current clamp mode at a stimulation frequency of 0.1Hz. Pclamp software and an Axon 200B patch clamp amplifier

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(Molecular Devices, Sunnyvale, CA) were used for the generation of voltage-/current-clamp protocols and data acquisition. To analyze current densities, membrane capacitance was measured using the analogue compensation circuit of the patch clamp amplifier. All current recordings were performed at room temperature (20-22°C). Cardiac action potentials, however, were recorded at near physiological temperatures of 30-32°C.

Data analysis. Data are expressed as mean ± SEM of n experiments or cells studied. Differences between means were usually tested using either a two-tailed Student's t-test or single factor analysis of variance followed by a two-tailed Dunnett's test to determine whether multiple treatment groups were significantly different from control. P-values <0.05 were considered statistically significant. Concentration-response relationships were fit to a Hill equation of the following form:

$$I_{drug}/I_{control} = 1 / (1 + [D/IC_{50}]^{n}),$$

where I indicates current or image densities, [D] is the drug concentration, n is the Hill coefficient and IC_{50} is the drug concentration necessary for half-maximal inhibition.

Results

Identification of cardiac glycosides as a compound class that reduces cell surface expression of hERG. To identify therapeutic compounds that may increase cardiac risk by reducing cell surface expression of hERG/I_{Kr}, we have developed a quantitative antibody-based chemiluminescent assay to monitor the heterologous expression of hERG channels at the cell surface using an HA epitope tag engineered into the extracellular loop spanning transmembrane domains S1 and S2 (hERG WT HA$_{ex}$;Wible et al., 2005). Using this assay, we exposed HEK293 cells stably expressing hERG WT HA$_{ex}$ overnight in a 96-well-format to increasing concentrations of 14 different cardiac glycosides. These included two clinically relevant drugs,

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digoxin and digitoxin, both of which are prototypical cardiac glycosides characterized by a steroid body, terminal sugar residues, and an unsaturated lactone ring at position C17. In addition, we tested cardiac glycosides without carbohydrate moieties (so-called '*genins*'), the compound pair ouabain/dihydroouabain which differs only in saturation of the lactone ring, and several natural glycosides including bufalin, proscillardin A and peruvoside. We found that 11 out of 14 cardiac glycosides used in our cell-based assay reduced cell surface expression of hERG with nanomolar affinity (Fig. 1 and Table1).

Evaluation of cardiac glycosides as hERG trafficking inhibitors using Western blots and current recordings. Arsenic trioxide and pentamidine both reduce hERG surface expression by inhibition of channel trafficking from the endoplasmic reticulum (ER) to the cell surface. To determine whether this is the case with cardiac glycosides, we performed Western blot experiments to test for changes at the protein level using three compounds: the two in clinical usage today, digoxin and digitoxin, which in patients reach plasma concentrations of 1-2 nM and 20-30 nM, respectively (Smith and Haber, 1970; Lehmann et al., 2000), as well as ouabain, which is used extensively as a research tool. In these experiments we exposed stably transfected HEK/hERG WT cells overnight to increasing concentrations of digoxin, digitoxin or ouabain. HERG is synthesized as an immature, ER resident protein of 135 kDa and as a fully-glycosylated protein of 155 kDa that is found at the cell surface (Zhou et al., 1998). In our experiments we show that overnight incubation with all three cardiac glycosides produced a concentration-dependent decrease in the amount of mature, fully-glycosylated cell surface protein, while the amount of ER resident hERG protein was either unaltered or increased at higher drug concentrations as a consequence of hERG trafficking inhibition (Fig. 2A). Production of the fully-glycosylated 155 kDa form of hERG was suppressed with IC_{50} values of

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18.1 ± 1.6, 27.8 ± 2.1 and 60.8 ± 6.2 nM for digitoxin, ouabain and digoxin, respectively (n=3, Fig. 2B).

To examine the functional consequences of hERG trafficking inhibition in HEK/hERG WT cells more directly, we recorded hERG currents from cells after overnight exposure to increasing concentrations of either digitoxin, digoxin, or ouabain. As expected from the Western blot data, we found that hERG currents were reduced by analyzing tail current amplitudes on return to –50 mV after maximal activation at +60 mV (Fig. 3A). The IC_{50} values measured were 11.1 ± 1.4 nM for digitoxin, 15.2 ± 0.9 nM for ouabain and 53.9 ± 14.3 nM for digoxin (n=3-11; Fig. 3B) which is similar to the dose-dependent reductions in fully-glycosylated, mature hERG protein determined from Western blots. To exclude that hERG currents are directly blocked by cardiac glycosides, we examined the effects of short-term (acute) extracellular application of digoxin and digoxigenin on hERG currents. HERG currents were activated by depolarizing pulses to +20mV from a holding potential of –80 mV and tail currents were measured on return to –50 mV in the absence and presence of 500 nM digoxin, which is about five times the concentration needed for half maximal block of channel trafficking. With 500 nM digoxin in the extracellular perfusate, we observed after 5 min on average an inhibition of 12.7 ± 5.8% (n=5), which was not different from current run-down observed over the same time period under control conditions (5 min; 8.4 ± 3.3%, n=5, Fig 3C). With 1000 nM of the aglygon digoxigenin, hERG tail currents decreased on average by 18.1 ± 2.5% (n=4) which was also not significantly different from control.

Is Na$^+$/K$^+$ ATPase inhibition required for hERG trafficking inhibition? As a class, cardiac glycosides are generally thought to bind to and inhibit Na$^+$/K$^+$ ATPase function with high potency and specificity. Nevertheless, considerable evidence exists that cardiac glycosides may

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also target sites other than the Na^+/K^+ ATPase. For example, cardiac glycosides directly activate

calcium release channels in the sarcoplasmic reticulum of the heart (Sagawa et al., 2002; Nishio

et al., 2004) and modulate proteins in the NF-κB signaling pathway (Srivastava et al., 2004).

Given this multiplicity of effects, we designed two experiments to directly address possible off-

target effects. In a first set of experiments we took advantage of an ouabain-resistant rat α1-

subunit encoded by the pCMV Ouabain[r] vector which we transiently transfected together with

hERG WT cDNA into HEK 293 cells. We then incubated co-transfected HEK 293 cells

overnight with increasing concentrations of ouabain and compared those transfections to

similarly treated cells which had been transfected with hERG WT and a pcDNA3 vector control.

We found that co-transfection of the ouabain resistant Na^+/K^+ ATPase subunit attenuated the

reduction of the fully, glycosylated cell surface form of hERG observed with ouabain (Fig 4A).

In a second set of experiments we inhibited the Na^+/K^+ pump of HEK 293 cells by using a

potassium-free extracellular solution (Orlov et al., 1999). In these experiments we incubated

stably transfected HEK/hERG WT cells overnight in defined, serum-free salt solutions with 5

mM (5K-sf, physiological control) and 2 mM $[K^+]_{ex}$ (2K-sf) or in a solution that was nominally

potassium free (0K-sf). On Western blots we found that overnight incubation with potassium-

free solution (0K-sf) reduced the amount of mature, fully-glycosylated cell surface protein by

90%, while 2 mM $[K^+]_{ex}$ did not alter expression of the cell surface form of hERG when

compared to controls incubated with 5 mM $[K^+]_{ex}$ (Fig 4B, C; n=3). The data from Western

blots were closely mirrored in electrophysiological current recordings performed in the presence

of 5 mM $[K^+]_{ex}$ to test for the functional effects of overnight incubation with potassium-free

solution (0K-sf). In these experiments maximal hERG tail current densities were significantly

reduced from 49.3 ± 6.8 pA/pF after overnight incubation in 5 mM $[K^+]_{ex}$ to 2.5 ± 0.8 pA/pF

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after incubation in nominally potassium-free solution (Fig 4D). After overnight incubation with

2 mM $[K^+]_{ex}$ tail current density averaged 53.3 ± 5.2 pA which was not significantly different

from controls incubated overnight in the presence of 5 mM $[K^+]_{ex}$ (Fig 4E; n=11-13; Dunnett's t-

test, $p < 0.05$). Taken together, our experiments suggest that hERG trafficking inhibition is

initiated by functional inhibition of plasmalemmal Na^+/K^+-ATPases and not another cellular

protein critically involved in hERG processing and trafficking.

Cardiac glycosides do not affect hKv1.5 and KCNQ1/KCNE1 trafficking. Since the

Na/K pump is critical for maintaining intracellular ion homeostasis, inhibition of the pump will

alter intracellular Na^+ and Ca^{2+} concentrations which could affect the processing and trafficking

of many different ion channel proteins in a non-specific manner. To address the specificity of

cardiac glycoside effects on hERG trafficking, we exposed stable cell lines constitutively

expressing either hKv1.5, a cardiac potassium channel expressed prominently in the atrium, or

KCNQ1/KCNE1 heteromultimers, encoding the cardiac slow delayed rectifier current I_{Ks}, to

increasing concentrations of digoxin and performed Western blots and/or electrophysiological

current recordings. After overnight incubation with 10, 100 or 300 nM digoxin, hKv1.5 was

detected on Western blots both as a core-glycosylated, immature ER form of about 68kDa and as

a mature, fully-glycosylated cell surface form of about 75kDa (Fig 5A) suggesting that long-term

exposure to digoxin did not alter the expression pattern of hKv1.5 (Fig 5B). In line with our

biochemical data, we found that hKv1.5 current densities were not significantly reduced upon

overnight incubation with 100 nM digoxin (Fig 4C). We determined current densities at the end

of depolarizing pulses to +20 mV and measured 738.9 ± 40.3 pA/pF under control conditions

and 740.9 ± 59.9 pA/pF on overnight exposure to 100 nM digoxin (n=10-11). Similarly, hKv1.5

currents were not affected by overnight incubation in nominally potassium-free solution (0K-sf)

to block Na/K pump function. When measured in electrophysiological experiments performed in the presence of 5 mM $[K^+]_{ex}$ current densities were 798.5 ± 73.3 pA/pF (at +20 mV) after overnight incubation with 5 mM $[K^+]_{ex}$ and 874.4 ± 71.1 pA/pF (at +20mV) after overnight exposure to potassium-free 0K-sf solution (n=12-13, supplemental Fig S1).

In addition, we recorded KCNQ1/KCNE1 currents in CHO cells under control conditions and after overnight exposure to 100 and 300 nM digoxin (Fig 6A). At the end of two second depolarizing pulses to +40 mV we measured current densities of 138.1 ± 25.5 pA/pF (n=10) under control conditions and of 97.2 ± 13.3 and 107.3 ± 22.5 pA/pF (n=9-10) on overnight exposure to 100 and 300 nM digoxin, respectively (Fig 6B). In a second set of experiments we inhibited the Na^+/K^+ pump by using a potassium-free extracellular solution and tested for effects on KCNQ1/KCNE1 current amplitudes. At the end of two second depolarizing pulses to +40 mV we measured in electrophysiological recordings performed in the presence of 5 mM $[K^+]_{ex}$ KCNQ1/KCNE1 current densities of 123.0 ± 13.5 pA/pF after overnight incubation in 5 mM $[K^+]_{ex}$ and 114.8 ± 18.3 pA/pF after incubation in nominally potassium-free solution (Fig 6C, D, n=9-14). Thus, neither hKv1.5 nor KCNQ1/KCNE1 were significantly affected by long-term exposure to digoxin or potassium-free extracellular solution supporting specificity of the cardiac glycoside-induced hERG trafficking block.

Evaluation of cardiac glycoside effects in guinea pig ventricular myocytes. Cardiac glycosides play an important role in the treatment of patients with congestive heart failure and/or atrial fibrillation (Gheorghiade et al., 2004), but their therapeutic use is associated with an increased susceptibility to cardiac arrhythmias (Ma et al., 2001; Grimard et al., 2005). To determine whether a reduction in cardiac hERG/I_{Kr} currents after long-term exposure to cardiac glycosides may contribute to the adverse cardiac events observed during therapy especially as

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result of intoxication (Roden, 1998), we cultured isolated guinea pig ventricular myocytes overnight in the presence of increasing concentrations of either digoxin or digitoxin. HERG/I_{Kr} currents were activated in cultured myocytes with depolarizing test pulses to +60 mV and isolated as E4031-sensitive tail currents on return to –40 mV (Fig.7A). On long-term exposure to digoxin hERG/I_{Kr} current density was significantly reduced from 0.66 ± 0.09 pA/pF measured in cultured control myocytes (n=10) to 0.32 ± 0.02 (n=6) and 0.15 ± 0.05 pA/pF measured in myocytes cultured in the presence of 30 and 100 nM digoxin, respectively (n=10; Dunnett's test, p<0.05; Fig 7B). On incubation with digitoxin we observed similar reductions in hERG/I_{Kr} current density from 0.68 ± 0.08 pA/pF in control myocytes to 0.42 ± 0.03 pA/pF at 30 nM and 0.21 ± 0.02 pA/pF at 100 nM digitoxin (n=5-6; Dunnett's test, p<0.05; Fig 7C). In marked contrast, two additional cardiac potassium currents, the slow delayed rectifier current I_{Ks} and the inward rectifier current I_{K1}, as well as cardiac calcium currents were not affected by long-term exposure to 100 nM digoxin (Fig 7D-7F).

Based on the long-term effects exerted by cardiac glycosides on hERG/I_{Kr} currents, we hypothesized that cardiac action potentials may similarly be prolonged after long-term but not short-term (acute) exposure to digitoxin. We focused on digitoxin because it reaches much higher plasma levels in patients (20-30 nM) than digoxin (1-2 nM). We studied short-term effects of extracellularly applied digitoxin on cardiac action potentials evoked in freshly isolated guinea pig ventricular myocytes at a stimulation frequency of 0.1 Hz. Current clamp experiments were performed at 30-32°C in the perforated patch configuration to preserve the intracellular milieu of native cardiomyocytes as much as possible since cardiac glycosides are known to induce complex changes in intracellular Na^+ and Ca^{2+} concentrations which depend critically on intracellular buffer capacities as well as the unimpeded function of several ion

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channel and ion transport proteins. We found that action potential duration was not affected

significantly by rapid application of 10 and 100 nM digitoxin with the extracellular perfusate.

Action potential duration was 367.4 ± 36.8 ms under control conditions and 374.2 ± 37.3 and

385.0 ± 44.7 ms when measured 5 min after start of perfusion with 10 or 100 nM digitoxin in the

bath (n=5, Fig 8A). Furthermore, we found that none of three major cardiac membrane currents,

I_{Kr}, I_{Ks} and I_{Ca} were blocked by fast extracellular application of 500 nM digitoxin, which was

consistent not only with our action potential recordings but also with experimental data reported

by others (e.g. Rocchetti et al., 2003; supplemental Table 1). To study long-term effects of

cardiac glycosides we cultured guinea pig ventricular myocytes overnight in the presence of

either 10 or 100 nM digitoxin and evoked action potentials using the perforated patch clamp

technique (Fig 8B). In these experiments action potential duration was significantly prolonged

from 296.3 ± 27.9 to 360.3 ± 47.6 and 607.2 ± 50.0 ms on incubation with 10 and 100 nM

digitoxin, respectively (n=4-6, Dunnett's, $p< 0.05$; Fig 8C). Thus, the possibility exists that

hERG trafficking inhibition may contribute to the increase in cardiac excitability seen with

certain cardiac glycosides in the clinic.

Discussion

The cardiac glycoside digoxin is an old and inexpensive medication frequently used in

the treatment of congestive heart failure with a major therapeutic benefit thought to be a positive

inotropic effect, i.e. an increase in contractility of the failing heart. The mechanism underlying

this inotropic effect is directly related to inhibition of Na^+/K^+ ATPase function by cardiac

glycosides that serves to increase intracellular $[Na^+]$ and is subsequently translated into elevated

intracellular Ca^{2+} concentrations via the Na^+/Ca^{2+} exchanger (Altamirano et al., 2006). It is now

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believed that changes in intracellular calcium handling are not only responsible for the inotropic effect produced by cardiac glycosides but contribute also to their considerable arrhythmogenic potential. In line with this hypothesis, ECG manifestations of digoxin toxicity include premature ventricular contractions most likely triggered by spontaneous SR Ca^{2+} release events due to an increased Ca^{2+} load of the sarcoplasmic reticulum (Hauptman and Kelly, 1999). Examples of cardiac arrhythmias precipitated by digoxin include ventricular bigeminy, atrial tachycardia and varying degrees of altered sinus and AV node function (Ma et al., 2001; Grimard et al., 2005) but typically not torsade de pointes arrhythmias (Fenichel et al., 2004), which together with QT prolongation are indicative for acLQTS. On the contrary, a shortening of the QT interval seems to be characteristic for patients with symptoms of cardiac glycoside toxicity (Cheng, 2004).

We were therefore surprised to find that a large set of 14 different cardiac glycosides reduced surface expression of hERG with high affinity in HEK293 cells, many at nanomolar concentrations. Western blots using clinically relevant digoxin and digitoxin as well as ouabain revealed that reduced surface expression was associated with a decrease in the fully glycosylated cell surface form of hERG. Thus, the dose-dependent reduction in surface expression of hERG protein and of hERG currents on long-term exposure to cardiac glycosides is the result of a drug-induced trafficking block. The effect was specific since neither heterologously expressed hKv1.5 nor KCNQ1/KCNE1 channels/currents were affected. Interestingly, three different experimental approaches suggested that hERG trafficking inhibition was initiated by functional inhibition of plasmalemmal Na^+/K^+-ATPases and not another cellular protein. First of all, overexpression of ouabain-insensitive rat Na^+/K^+-ATPase subunits attenuated the effect of ouabain as a hERG trafficking inhibitor. Secondly, inhibition of Na/K pumps with nominally potassium free extracellular solution mimicked the effects of cardiac glycosides on hERG

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trafficking. Finally, we observed a good correlation between hERG trafficking inhibition and the published inhibition of the enzymatic Na^+/K^+-ATPase activity by a diverse set of cardiac glycosides. Proscillaridin A, peruvoside, nerifollin, ouabain and digitoxin reduce hERG cell surface expression and bind to/inhibit Na/K ATPase with similar affinity (Paula et al, 2005). Bufalin, however, the most potent inhibitor of hERG surface expression (Table 1), binds to Na^+/K^+ ATPase with only moderate affinity. This disconnect may be resolved by the observation that bufalin shows much higher potency in assays that monitor the functional inhibition of Na^+/K^+ ATPase activity rather than drug binding (Paula et al., 2005). A close correlation can also be seen in cardiac glycosides without carbohydrate moieties. Loss of sugar residues not only reduces the binding affinity of the respective molecules by one order of magnitude but also decreases their potency as inhibitors of hERG surface expression. Likewise, saturation of the lactone ring in the compound pair ouabain/dihydroouabain causes a reduced potency in our cell based surface expression assay as well as in Na^+/K^+ ATPase binding/inhibition assays. Overall, most major trends that have been observed with respect to binding affinity and inhibitory potency as a consequence of structural modification of cardiac glycosides seem to be mirrored in our cell surface expression assay. This suggests that hERG trafficking inhibition by cardiac glycosides does not occur via direct interaction with hERG or any closely associated protein necessary for folding or export of hERG from the ER. The specific mechanism coupling inhibition of the Na/K pump to hERG processing and trafficking in the ER, however, is currently unknown.

Since Na^+/K^+-ATPases are considered the main physiological target for the clinically important cardiac glycoside digoxin which inhibits hERG trafficking with nanomolar affinity, it is puzzling why toxicity in patients has rarely been associated with QT prolongation and/or

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torsade de pointes arrhythmias (Tan et al, 1998). The simplest explanation may be that plasma concentrations of digoxin typically do not reach levels high enough to inhibit hERG trafficking in patients. We found in guinea pig ventricular myocytes that hERG/I_{Kr} current density was half-maximally reduced by about 30 nM digoxin while three other major cardiac membrane currents were not affected by up to 100 nM. In fact, digoxin plasma concentrations measured in patients range only from 1-2 nM and rarely exceed 3-5 nM even under conditions of severe intoxication (Smith and Haber, 1970). Thus, clinically relevant digoxin concentrations are not likely to affect hERG trafficking.

The situation is different for digitoxin, another cardiac glycoside in use for the treatment of heart failure patients in Europe (Lehmann et al., 2000; Belz et al., 2001; Haux et al., 2001). Digitoxin has a much longer half-life than digoxin, and much higher plasma concentrations, in the range of 20-30 nM, are reached during therapy and exceeded under conditions of intoxication (Lehmann et al., 2000). In our experiments 30 nM digitoxin reduced hERG/I_{Kr} current density in guinea pig myocytes by half and prolonged cardiac action potential duration. The fact that digitoxin concentrations for half-maximal inhibition of hERG/I_{Kr} currents are somewhat higher in cardiomyocytes than in HEK/hERG cells (30 nM versus 11 nM, respectively) may be explained best by expression of different Na/K pump isoforms or isoform ratios in these two preparations and possibly also by the specific subcellular localization of different Na/K isoforms in cardiomyocytes (Wasserstrom and Aistrup, 2005). Regardless, there is the distinct possibility that hERG trafficking is inhibited by therapeutic concentrations of digitoxin used in patients. Just like digoxin, however, digitoxin has usually not been associated with QT prolongation and torsade de pointes arrhythmias, the electrocardiographic hallmarks of acLQTS in the clinic. The reason may be that digitoxin exerts multiple, additional effects on heart function including

changes in intracellular calcium handling as well as neurohumoral changes that alter the functional status of the autonomic nervous system, all of which may contribute to a more complex set of electrical changes and arrhythmias than expected for a simple reduction of hERG currents.

In addition to their value as cardiac drugs, cardiac glycosides have well known anti-proliferative properties. In particular digitoxin is considered a potent anti-cancer drug effective not only in tumor cell lines (Lopez-Lazaro et al., 2005; Kometiani et al., 2005) but possibly also in patients where anti-proliferative effects of cardiac glycosides have been reported for breast cancer, leukemia/lymphomas and cancer of the kidney/urinary tract (Haux et al., 2001). Because hERG channels are highly expressed in many tumor cells and are thought to control cell proliferation as well as invasiveness, down-regulation of hERG surface expression may facilitate the anti- proliferative actions described for cardiac glycosides (Arcangeli, 2005). Furthermore, digitoxin may also be useful to suppress inflammation in cystic fibrosis via inhibition of the NF-κB pathway (Srivastava et al., 2004) or the cytotoxicity of poly-glutamine-expanded proteins (Piccioni et al., 2004). Consequently, digitoxin has been used more recently as a lead compound for drug development (Langenhan et al., 2005). In addition, natural glycoside compounds such as bufalin or resibufogenin, which are major components of the traditional Chinese medicine chan'su, are being explored as potential drugs, all of which may be burdened by ab initio cardiotoxicity due to hERG trafficking inhibition (Kawazoe et al., 1999; Xie et al., 2001).

In summary, cardiac glycosides, together with two previously described hERG trafficking inhibitors, arsenic trioxde and pentamidine (Ficker et al., 2004; Kuryshev et al., 2005; Cordes et al., 2005), comprise a growing group of compounds that increase cardiac risk not via direct hERG block but indirectly via trafficking inhibition. The significance of drug-induced

trafficking inhibition for the development of novel therapeutic compounds is further highlighted

by drugs such as celastrol, which belongs to an even larger group of compounds that both block

hERG directly and at the same time inhibit its processing and trafficking (Wible et al., 2005; Sun

et al., 2006). Even though our observations have no immediate impact on the use of digoxin in

the treatment of heart failure and atrial fibrillation, the therapeutic use of digitoxin may be more

problematic since hERG trafficking inhibition can contribute to the proarrhythmogenic potential

of this particular cardiac glycoside. Finally, the cardiotoxicity of digitalis glycosides contained

in natural compounds such as bufalin that inhibit hERG trafficking with very high potency has to

be of some concern since natural compounds are regulated less stringently on the market place

than 'classical' drugs developed by pharmaceutical companies. Overall, the growing number of

hERG trafficking inhibitors poses a novel problem for the identification of compounds that

impair hERG channel function in pre-clinical safety studies that are geared towards detection of

acute channel block.

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Footnotes

a) This work was supported by National Institute of Health grant HL71789 (to E.F.) and

CA106028 (to B.A.W.).

b) B.A.W. is an employee of ChanTest Inc., a company that provides hERG trafficking assays as

part of their services.

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Legends for Figures

Fig.1. Effects of cardiac glycosides on surface expression of hERG. Overnight treatment with cardiac glycosides reduces surface expression of HA_{ex}-tagged hERG protein stably expressed in HEK293 cell in a concentration dependent manner. Surface expression levels were determined by chemiluminesence measurements and normalized relative to untreated controls. Shown are concentration-response curves for seven different cardiac glycosides. Concentration-response data were prepared from at least three independent assays and fitted to Hill equations with the Hill coefficient kept constant at 3. IC_{50} values for all cardiac glycosides tested in the hERG surface expression assay are shown in Table 1.

Fig. 2. Long-term exposure to cardiac glycosides inhibits maturation of hERG protein. A, Western blots showing effects of overnight treatment with increasing concentrations of digoxin and digitoxin on hERG stably expressed in HEK293 cells. Both cardiac glycosides reduced the fully-glycosylated, mature 160 kDa form of hERG (fg) in a concentration dependent manner. (cg) indicates core-glycosylated ER resident 135 kDa form of hERG. B, concentration-dependent reduction of fully-glycosylated, 160 kDa form of hERG after long term-exposure to digitoxin, ouabain or digoxin. Image densities on Western blots were quantified using a Storm PhosphorImager and normalized to untreated controls. Data were fitted to Hill equations with the Hill coefficient kept constant at 3. IC_{50} was 18.1 ± 1.6 nM for digitoxin, 27.8 ± 2.06 nM for ouabain and 60.8 ± 6.2 nM for digoxin (n=3).

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Fig. 3. Long-term exposure to cardiac glycosides reduces hERG currents stably expressed in HEK293 cells. A, ensemble of hERG currents recorded after overnight culture (24h) under control conditions or with exposure to 100 nM digoxin (24h). Currents were elicited using depolarizing voltage steps from –60 to +60 mV in 10 mV increments. Holding potential was –80 mV. Tail currents were recorded on return to –50 mV. Dashed lines indicate zero current level. B, concentration dependent reduction of normalized hERG tail current densities after overnight exposure to digitoxin, ouabain or digoxin. Data were fitted to Hill equations with the Hill coefficient kept constant at 4. IC_{50} was 11.1 ± 1.4 nM for digitoxin, 15.2 ± 0.9 nM for ouabain and 53.9 ± 14.3 nM for digoxin (n=3-11). C, hERG currents recorded under control conditions and after bath application of 500 nM digoxin or 500 nM E4031. Currents were elicited from a holding potential of –80 mV with depolarizing test pulses to +20 mV. Tail currents were recorded upon return to –50 mV. The inhibition observed with 500 nM extracellularly applied digoxin was not different from the current run-down observed under control conditions.

Fig. 4. HERG trafficking inhibition by cardiac glycosides is coupled directly to Na^+/K^+ ATPase inhibition. A, Western blot showing effects of overnight treatment with increasing concentrations of ouabain on hERG protein transiently expressed in HEK293 cells and on co-transfection with a rat ouabain-insensitive Na/K ATPase (pCMV Ouabain[r]). Co-expression of pCMV Ouabain[r] attenuated ouabain-induced hERG trafficking inhibition. B, Western blot showing effects of 5mM (5K-sf), 2mM (2K-sf) and nominally K-free extracellular solution (0K-sf) on hERG maturation. C, image densities of fully glycosylated (fg) hERG protein bands quantified as a function of extracellular potassium concentrations using a PhosphorImager.

Image densities were normalized to the fully-glycosylated 155 kDa protein form of hERG measured in the presence of 5mM $[K]_{ex}$ (n=3). D, representative hERG currents recorded after overnight culture in the presence of 5mM $[K]_{ex}$ or after long-term exposure to a nominally potassium free external solution (0K-sf). Currents were elicited in the presence of 5 mM $[K]_{ex}$ from a holding potential of –80 mV with depolarizing test pulses to +60 mV in 10mV increments. Tail currents were recorded on return to –50 mV. Dash to left of current traces indicates zero current level. E, quantification of hERG current densities measured after overnight culture in the presence of 5mM $[K]_{ex}$, 2mM $[K]_{ex}$ and in a nominally potassium free solution (0K). Current densities were measured as maximal tail current amplitudes in the presence of 5 mM $[K]_{ex}$ on return to –50mV and normalized to cell capacitances. Current densities were significantly different between 5mM $[K]_{ex}$ control and cells exposed to 0K (n=11-13; Dunnett's t-test, $p < 0.05$).

Fig. 5. Digoxin does not alter expression of hKv1.5. A, Western blot showing hKv1.5 protein stably expressed in L-cells after overnight exposure to increasing concentrations of digoxin. HKv1.5 is expressed as a core-glycosylated protein of 68 kDa (cg) and a fully-glycosylated, mature protein of 75 kDa (fg). B, image densities of fully glycosylated and core-glycosylated hKv1.5 protein bands quantified as function of digoxin concentration using a PhosphorImager. All image densities were normalized to the fully-glycosylated 75 kDa protein form of hKv1.5 measured under control conditions (n=3). C, representative hKv1.5 currents recorded after overnight culture under control conditions or after long-term exposure to 100 nM digoxin. Cells were held at –80 mV, test pulses were applied form –70 to +40 mV in increments of 10mV. Tail

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currents were recorded on return to –100 mV. Dash to left of current traces indicates zero

current level. D, quantification of hKv1.5 current densities measured after overnight culture

under control conditions or in the presence 100nM digoxin. Current densities were measured at

the end of a depolarizing test pulse to +20 mV and normalized to cell capacitance. Current

densities were not significantly different between control and digoxin treated cells (n=10-11).

Fig. 6. Both digoxin and incubation in 0K solution do not alter expression of

KCNQ1/KCNE1 channels. A, representative current recordings of KCNQ1/KCNE1 channels

stably expressed in CHO cells and cultured overnight under control conditions or in the presence

of 300nM digoxin. Currents were elicited from a holding potential of –80 mV with 2 s test

pulses from –60 to +60 mV in 20 mV increments. Tail currents were recorded on return to –50

mV. Dash to the left indicates zero current level. B, quantification of current densities measured

in cells cultured overnight under control conditions or in the presence of either 100 or 300 nM

digoxin. Current densities were determined from current amplitudes at the end of depolarizing

test pulses to +40 mV and normalized to cell capacitance. Current densities measured in digoxin

treated cells were not significantly different form control cells (n=9-11). C, representative

current recordings of KCNQ1/KCNE1 channels stably expressed in CHO cells and cultured

overnight either in the presence of 5mM $[K]_{ex}$ (5K-sf) or in a nominally potassium-free external

solution (0K-sf). Currents were elicited in the presence of 5 mM $[K]_{ex}$ from a holding potential

of –80 mV with 2 s test pulses from –60 to +60 mV in 20 mV increments. Tail currents were

recorded on return to –50 mV. Dash to the left indicates zero current level. D, quantification of

current densities measured in cells after overnight culture in the presence of 5mM $[K]_{ex}$, and in a

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nominally potassium-free solution (0K). Current densities were determined in the presence of 5

mM $[K]_{ex}$ from current amplitudes at the end of depolarizing test pulses to +40 mV and

normalized to cell capacitance. Current densities measured in 0K cells were not significantly

different form controls (n=9-14).

Fig. 7. Long-term exposure to cardiac glycosides reduces native hERG/I_{Kr} currents in

cultured guinea pig ventricular myocytes. A, representative E4031-sensitive hERG/I_{Kr}

currents recorded in a guinea pig ventricular myocyte cultured overnight under control

conditions or in the presence of 100 nM digoxin. HERG/I_{Kr} currents were isolated using digital

subtraction of current traces recorded in the absence and presence of 5 μM E4031. Raw current

traces were elicited with a depolarizing voltage step to +60 mV from a holding potential of –40

mV. B, quantification of hERG/I_{Kr} current densities measured as E4031-sensitive tail currents

on return to –40 mV in guinea pig ventricular myocytes cultured overnight under control

conditions or in the presence of 30 and 100 nM digoxin. I_{Kr} current density was reduced in a

concentration-dependent manner (n=6-10). C, reduction in hERG/I_{Kr} tail current densities on

overnight exposure to 30 or 300 nM digitoxin (n=5-8). D, quantification of I_{Ks} current densities

measured in guinea pig ventricular myocytes cultured overnight under control conditions or in

the presence of 100 nM digoxin (n=8). I_{Ks} tail currents were measured on return to –40 mV in

the presence of E4031 following upon a depolarizing test pulse to +60 mV. I_{Ks} current density

was 3.9 ± 0.5 pA/pF under control conditions and 4.1 ± 0.6 pA/pF on exposure to 100nM

digoxin (n=8; not significantly different, Student's t-test). E, quantification of I_{K1} current

densities in myocytes cultured under control conditions (-38.8 ± 4.3 pA/pF) or in the presence of

100 nM digoxin (-41.9 ± 5.5 pA/pF; n=8; ns, Student's t-test). I_{K1} currents were measured at the

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end of hyperpolarizing test pulses to –100 mV from a holding potential of –40 mV. F, averaged

IV-relationships of cardiac calcium currents recorded in ventricular myocytes cultured overnight

under control conditions or after exposure to 100nM digoxin. Raw current traces were elicited

using 300 ms step depolarizations from -30 to +60 mV in increments of 10mV. Holding

potential was –40 mV. Peak current density measured at + 20mV was –5.2 ± 0.3 pA/pF under

control conditions and –5.1 ± 0.2 pA/pF on overnight exposure to 100nM digoxin (n=8, ns,

Student's t-test).

**Fig. 8. Long-term exposure to digitoxin prolongs the cardiac action potential in isolated
guinea pig ventricular myocytes.** A, quantitative analysis of action potential duration (APD)

on short term exposure of freshly isolated guinea pig ventricular myocytes to 10 or 100nM

digitoxin. APD was not significantly different in digitoxin-treated and control myocytes (n=5).

  B, representative current clamp recordings of action potentials elicited in guinea pig ventricular

myocytes cultured overnight under control conditions or in the presence of 10 and 100nM

digitoxin. C, quantitative analysis of APD measured in guinea pig ventricular myocytes cultured

overnight under control conditions and in the presence of either 10 or 100nM digitoxin. APD

was significantly prolonged in myocytes cultured overnight in the presence of 100nM digitoxin.

(n=4-6; Dunnett's test, $p < 0.05$). All action potentials were recorded at 30-32°C using the

perforated patch clamp technique.

Table 1

IC$_{50}$ values for inhibition of hERG surface expression by the entire set of cardiac glycosides used in our experiments. Half-maximal inhibition of hERG surface expression was determined from experiments as shown in Figure 1. Ouabagenin did not affect hERG surface expression in the concentration range studied (NA). Cardiac glycosides depicted both in Table 1 and in Figure 1 are numbered similarly.

Cardiac glycosides	IC$_{50}$ [nM]
Bufalin	1.8 ± 0.2
Proscillaridin A	2.0 ± 0.3
Peruvoside	3.5 ± 0.1
Neriifolin	4.9 ± 0.4
Ouabain, 1	9.8 ± 0.3
Digitoxin, 2	13.9 ± 2.5
Lanatoside C	60.2 ± 4.1
Digitoxigenin, 3	62.8 ± 9.4
Digoxin, 4	73.1 ± 11.4
Dihydroouabain, 5	194.6 ± 28.0
Digoxigenin, 6	206.3 ± 30.8
Strophanthidol	>300
Gitoxigenin	>2000
Ouabagenin, 7	NA

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

Fig 6

Fig 7

Fig 8

A

short-term

digitoxin: con, 10nM, 100nM

B

overnight

100nM digitoxin (24h)
10nM digitoxin (24h)
control (24h)

C

overnight

*

digitoxin: con, 10nM, 100nM