

Amsacrine modifies action potential configuration without its prolongation via the blockade of multiple ion currents in canine ventricular cells

József Óvári^{a,b,1}, Zsigmond Máté Kovács^{a,1}, János Magyar^{a,c}, Tamás Bányász^a, Péter P. Nánási^{a,d}, Balázs Horváth^a, Zoltan Varga^e, Norbert Szentandrassy^{a,f,*}

^a Department of Physiology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

^b Doctoral School of Dental Sciences, University of Debrecen, Debrecen, Hungary

^c Division of Sport Physiology, Department of Physiology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

^d Department of Dental Physiology and Pharmacology, Faculty of Dentistry, University of Debrecen, Debrecen, Hungary

^e Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

^f Department of Basic Medical Sciences, Faculty of Dentistry, University of Debrecen, Debrecen, Hungary

ARTICLE INFO

Keywords:

Amsacrine

Canine cardiomyocytes

Action potential

I_{Na}

$I_{Ca,L}$

I_{to}

ABSTRACT

Amsacrine is an antineoplastic agent for acute lymphoblastic leukemia. It contains methanesulfonamide group—similar to hERG/ I_{Kr} inhibitors like E-4031 and dofetilide—therefore may exert cardiac arrhythmias. Although amsacrine inhibits expressed hERG channels, its effects on native cardiac electrophysiology remain unclear. We investigated the effects of 1 and 10 μ M amsacrine on action potentials (APs) and underlying ionic currents in isolated canine ventricular cardiomyocytes. Amsacrine reduced phase 0 parameters: maximal upstroke velocity ($\sim$ 50–60%), action potential amplitude ($\sim$ 15%), and peak potential ($\sim$ 55%), suggesting inhibition of sodium current. Moreover, the maximal rates of phase 1 and 3 repolarizations, and plateau potentials were reduced, suggesting effects on multiple ionic currents. Despite these, AP duration (APD_{20} – APD_{90}) remained unaffected. AP voltage-clamp analysis revealed a complex amsacrine-sensitive current consisting of an early inward component consistent with sodium current inhibition, a second early outward component suggesting transient outward current suppression, a mid-plateau inward component likely reflecting inhibition of inward plateau currents, and a late outward component compatible with I_{Kr} inhibition. 10 μ M amsacrine reduced L-type calcium, transient outward potassium and expressed $Na_v1.5$ channel currents by 22, 38, and 10%, respectively. Native I_{Kr} was also reduced in a concentration-dependent manner (152 nM IC_{50} and 1.21 Hill coefficient). These findings demonstrate that amsacrine modulates multiple cardiac ion currents. The lack of AP prolongation despite I_{Kr} inhibition can be due to concomitant suppression of inward currents. As the tested concentrations overlap with therapeutic plasma levels (3–18 μ M), amsacrine may carry a non-negligible risk of cardiac electrophysiological effects, particularly in susceptible patients.

1. Introduction

Amsacrine is a chemotherapeutic agent used for the treatment of acute myeloid leukemia [1]. It has been reported to cause hypomagnesemia and QT prolongation together with side effects affecting gastrointestinal system, liver functions and central nervous system [2,3]. Shinar and Hasin reported transient QT prolongation (observed after and on the day of the infusion but not later) in all 12 patients in all 12 leads without changes in PR, and QRS durations and heart rate [4]. In some cases QT prolongation persisted and developed at each amsacrine

infusion [5]. Cardiac side effects of prolonged application was also reported in the form of cardiomyopathy or congestive failure but the overall incidence of amsacrine-related cardiotoxicity was low (1%) in 683 patients [6]. The incidence of cardiotoxic side effect of amsacrine was very variable, some patients who received high doses (200 mg/m² daily for 3–5 days) showed no cardiac problems at all, but even sudden cardiac death was observed in a few cases [6]. In conclusion authors stated that although amsacrine can clearly lead to QT prolongation, T wave inversion, sometimes atrial and ventricular arrhythmias but serious cardiac toxicity is uncommon. Previous anthracycline treatment,

* Correspondence to: Department of Physiology, University of Debrecen, P.O. Box 22, Debrecen H-4012, Hungary.

E-mail address: szentandrassy.norbert@med.unideb.hu (N. Szentandrassy).

¹ The first two authors have equal contributions to the work.

hypokalemia [7] and existing cardiac problems can however increase the development of cardiac side effects [6]. Nevertheless, amsacrine was placed on the CredibleMeds website in 2018 with a note of conditional risk of TdP [8].

There are only a few animal data about the effects of amsacrine. In rabbits, ECG abnormalities were seen in form of A-V blocks, ventricular premature contractions, supraventricular tachycardia, atrial flutter, intraventricular conduction delay, and ventricular tachycardia on top of the negative inotropic effect [9]. Another rabbit study however reported no effect of amsacrine [10]. In rat hearts, the negative inotropy was confirmed and the depression in excitability and prolongation of refractoriness was also observed [11]. The long term cardiotoxicity was however not detected in rats [12]. In dogs, a study reported A-V block, QRS and QT interval prolongation, marked ST-T wave changes when high dose amsacrine was administered, moreover, one animal died presumably due to cardiac arrest [13].

The mechanism behind the aforementioned effects is not examined in detail, except the study of Thomas et al., where amsacrine-induced inhibition of hERG channels expressed in *Xenopus* oocytes and HEK cells were observed in the open and inactivated states of the channels which explains the QT prolongation [14]. Apart from that report, according to our knowledge, no data is available so far on the electrophysiological actions of amsacrine on ion channels or the cardiac action potential. Amsacrine's chemical structure contains a methanesulfonamide group (Fig. 1) similarly to those of known blockers of the I_{Kr} , such as E-4031 and MK-499, D-sotalol and dofetilide [15]. The native current based on $K_{v11.1}$ or better known as hERG channels, is a main repolarizing current of the ventricular AP. The amsacrine-induced I_{Kr} reduction can lead to congenital type 2 long QT syndrome [16,17] and due to the development of early afterdepolarizations (EADs) [18] might lead to a potentially lethal torsade de pointes (TdP) type of ventricular arrhythmia [16].

Therefore, the aim of the present study was to characterize the electrophysiological effects of amsacrine on cardiac action potentials and the underlying ionic currents in canine ventricular cardiomyocytes.

Our key findings include the lack of amsacrine-induced AP prolongation of canine ventricular cardiomyocytes in therapeutic concentration despite significant reduction of maximal rates of AP phases 0, 1, and 3, as well as AP peak, AP, Plateau₂₀ and Plateau₅₀ amplitude values. These AP changes were supported by properties of the amsacrine-sensitive current obtained with AP voltage clamp and direct reduction of multiple currents.

2. Materials and methods

2.1. Isolation of single canine ventricular myocytes

Adult male and female mongrel dogs were anesthetized with

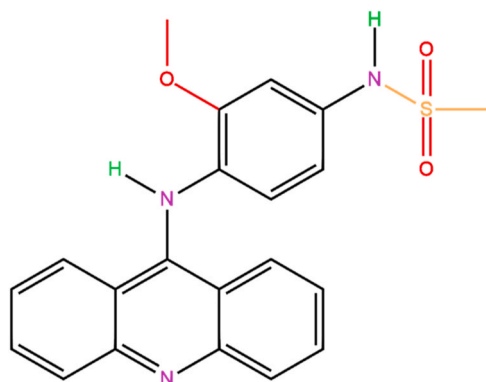


Fig. 1. Chemical structure of amsacrine, created by ChemDrawPro 12.0 software.

intramuscular injections of 10 mg/kg ketamine hydrochloride (CP-Ketamin, Produlab Pharma BV, Raamsdonksveer, The Netherlands) + 1 mg/kg xylazine hydrochloride (CP-Xylazin, Produlab Pharma BV, Raamsdonksveer, The Netherlands) according to a protocol approved by our local ethical committee (license No: 2/2020/DEMÁB) and all procedures conformed to the principles outlined in the Declaration of Helsinki in 1964. We isolated cells with enzymatic digestion using the segment perfusion technique as described previously [19].

2.2. Recording of action potentials with conventional microelectrode technique

Rod-shaped viable cells with clear striations and edges were measured with 20–30 MΩ conventional sharp microelectrodes (filled with 3 M KCl) at 37 °C, where action potentials (APs) were continuously stimulated at 1 Hz applied at 120% of threshold amplitude with a DSR4 type stimulator (Főnixcomp Kft., Debrecen, Hungary) as described previously [19]. APs were recorded with Multiclamp-700A or 700B amplifiers (Molecular Devices, LLC., San Jose, CA, USA) in current-clamp mode with pClamp10.0 software (Molecular Devices, LLC., San Jose, CA, USA) and digitized with Digidata 1440 A (Molecular Devices, LLC., San Jose, CA, USA) at 50 kHz. AP parameters are stable for the period of our experimental protocol since the cytosol was not dialyzed [20]. Cells were perfused with bicarbonate buffer containing Tyrode solution (BTY) containing (in mmol/L): NaCl 121; KCl 4; MgCl₂ 1; CaCl₂ 1.3; HEPES 10; glucose 10; NaHCO₃ 25; (pH=7.3; adjusted with NaOH). APs were analyzed off-line from ten consecutive APs, and the following parameters were calculated and averaged: APD₂₀, APD₅₀, APD₇₅, and APD₉₀ values (duration of the AP from the peak to 20, 50, 75, and 90% of repolarization, respectively), maximal rate of voltage change in phase 0, 1 and 3 (V_{max}, V_{ph1max}, and V_{max}, respectively), resting membrane potential (RMP), AP peak, APA (action potential amplitude, determined as the difference between peak and RMP) and MP difference between the RMP and the MP values at the 20 and 50% duration of APD₉₀ (Plateau₂₀ and Plateau₅₀ amplitudes).

Short-term variability (SV) of APD was calculated from 50 consecutive APs as follows:

$$SV = \frac{\sum_{i=1}^n (|APD_{i+1} - APD_i|)}{n\sqrt{2}}$$

where SV is short-term variability, APD_i and APD_{i+1} indicate the APD₉₀ values of the *i*th and *i* + 1th APs, respectively, and *n* denotes the number of consecutive beats analyzed [21,22].

Cells were perfused with 1 or 10 μM amsacrine for 8–9 min to reach steady-state effect then followed by a 12–13-min-long period of washout. Amsacrine hydrochloride (Merck, product: A9809) was dissolved to 1 and 10 μM concentrations from 10 mM stock prepared in DMSO. The maximal DMSO concentration (in case of 10 μM amsacrine) was 0.1%. As shown previously, even 1.5% DMSO did not modify any parameters of the canine left ventricular AP [19].

2.3. Voltage clamp studies

Cells were continuously perfused at 37 °C with BTY solution (see above for composition). Borosilicate glass suction pipettes had tip resistance of 2–3 MΩ after filling with pipette solution containing (in mmol/L): K-aspartate 120, KCl 30, MgATP 3, HEPES 10, Na₂-phosphocreatine 3, EGTA 0.01, cAMP 0.002, KOH 10 at pH = 7.3 in AP voltage clamp and with KCl 110, KOH 40, HEPES 10, EGTA 10, TEACl 20, K-ATP 3 at pH = 7.2 in L-type calcium current (*I*_{Ca,L}) recordings. After establishing high (1–10 GΩ) resistance seal by gentle suction, the cell membrane beneath the tip of the electrode was disrupted by further suction and/or by applying 1.5 V electrical pulses for 1 ms. The series resistance was typically 4–8 MΩ. We discarded the experiments where series resistance was high or substantially increased during the measurement.

Current signals were recorded with Multiclamp–700A or 700B amplifiers (Molecular Devices, LLC., San Jose, CA, USA) in voltage-clamp mode with pClamp10.0 software (Molecular Devices, LLC., San Jose, CA, USA) and digitized with Digidata 1440 A (Molecular Devices, LLC., San Jose, CA, USA). in voltage-clamp mode at 50 or 10 kHz in AP voltage-clamp and conventional voltage clamp recordings, respectively. We determined cell capacitance using short hyperpolarizing pulses to -10 mV for 45 ms from 20-ms-long depolarization to 0 mV from the holding potential of -80 mV applied at 10 Hz. Average value of cell capacitance was 154.1 ± 7.2 pF in the 34 patch-clamped myocytes.

AP voltage-clamp experiments were conducted according to the method described previously [19,23] after recording each cell's own AP. A previously recorded typical AP (recorded with 700-ms-long cycle length pacing on a midmyocardial cell, termed as “canonic” AP) was applied to the voltage clamped cells in whole-cell configuration as command signal. Amsacrine-sensitive current was obtained by subtracting the current signals recorded in the presence of 1 or 10 μ M amsacrine from those measured in control condition (BTY). Ion currents were normalized to cell capacitance values to calculate current densities.

Conventional voltage-clamp experiments were conducted to measure the L-type calcium current and transient outward potassium currents in whole cell configuration according to the method used previously [24]. The series resistance was continuously compensated to 70% during these experiments. $I_{Ca,L}$ was recorded during depolarizations to $+5$ mV arising from the holding potential of -40 mV in every 5 s. In these experiments the perfusion solution contained 3 mM 4-aminopyridine, 1 μ M E4031, and 1 μ M HMR–1556 in order to block K^+ currents (I_{To} , I_{Kr} and I_{Ks} , respectively).

I_{To} was evoked by 200 ms-long depolarizations to $+60$ mV arising from the holding potential of -80 mV in every 3 s. Before each test pulse, a short (5 ms) depolarization to -40 mV was applied in order to inactivate the fast Na^+ current. The external solution contained 1 μ M E4031 and 5 μ M nifedipine to block I_{Kr} and Ca^{2+} currents, respectively.

I_{Kr} was recorded as tail currents upon repolarization to -40 mV after a 500 ms long depolarization to $+40$ mV elicited in every 15 s from the holding potential of -40 mV. The external solution contained 30 μ M Chromanol293B and 5 μ M nifedipine to block other potassium (I_{Ks} and I_{To}), and Ca^{2+} currents, respectively.

The effect of amsacrine was tested on human $Na_v1.5$ channels expressed on CHO cells exactly as described previously [25]. Recovery from inactivation was assessed by the standard twin-pulse protocol increasing the interpulse interval spent at the holding potential of -100 mV between the two 50-ms-long depolarizations to 0 mV.

2.4. Statistics

Data are presented as the arithmetic means $\pm$ Standard Error of the Mean (SEM) of the studied parameters. Given the biological variability among myocardial cells, we treated each cell as independent in the statistical tests. Statistical significance of differences was evaluated using one-way ANOVA and Tukey post-hoc test when we compared BTY, amsacrine and washout if the values in all three groups had normal distribution, otherwise Kruskal-Wallis (K-W) and Dunn post-hoc test was used. Paired *t*-test or Wilcoxon signed-rank test (as appropriate based on the distribution) was used when comparing amsacrine with BTY only (and not considered the washout). Unpaired *t*-test, Welch, or Mann-Whitney test was applied according to the distribution (normal or not) and the variance of the results when 1 and 10 μ M amsacrine-induced effects were compared. Results were also reported in the form of median $\pm$ interquartile range in cases of non-normally distributed AP parameters in [supplementary tables](#). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Effect of 1 μ M amsacrine on canine left ventricular cells

Based on the previously published IC_{50} values of amsacrine in expressed cell lines (0.2 and 2 μ M in HEK cell and *Xenopus* oocytes) [14], we first examined the actions of 1 μ M amsacrine on the action potential of left ventricular myocytes. This concentration inhibits approximately 80% of the hERG current in HEK cells [14]. In these experiments amsacrine perfusion lasted for 8–9 min to ensure steady-state conditions, then we tested the reversibility of any potential effects with an approximately 12–13-min-long washout period. Unexpectedly, no significant change was observed in AP durations (Table 1, Fig. 2A). Depolarization was reduced by 1 μ M amsacrine as indicated by the reduction of all three examined AP parameters related to this phase. Maximal rate of depolarization, $V + \max$ decreased by $46 \pm 12\%$ (Fig. 2A–B). AP amplitude was reduced by $15 \pm 4\%$ (Fig. 2C), whereas AP peak reduction was even bigger ($54 \pm 12\%$) (Fig. 2D). These changes occurred despite an increase in stimulation current amplitude to maintain suprathreshold excitation. It must be highlighted that in case of two cells where the AP peak has been reduced below 0 mV in the presence of amsacrine, all values below 0 mV were set to 1 mV for analysis purposes. This adjustment, however, did not affect statistical comparisons. Upon washout, these amsacrine-induced changes were mostly reversible indicated by the lack of significant difference between BTY and washout (Fig. 2).

The following AP parameters were significantly different only if we compared them between BTY and amsacrine (and not considering washout) (Fig. 3). The maximal rate of phase 1 repolarization, V_{Ph1max} was reduced by $55.8 \pm 9.2\%$ (Fig. 3A), $V - \max$ was also reduced (by $5.5 \pm 2.6\%$) (Fig. 3B), similarly to Plateau₂₀ amplitude ($3.5 \pm 1.4\%$ reduction) (Fig. 3C), and Plateau₅₀ amplitude ($6.1 \pm 2.2\%$ reduction) (Fig. 3D). In contrast, APD₂₀ was increased in the presence of 1 μ M amsacrine ($30.6 \pm 9.7\%$ increase), not shown.

Several further AP parameters were not altered by 1 μ M amsacrine (Table 1).

3.2. Effect of 10 μ M amsacrine on canine left ventricular cells

Next we studied the effects of 10 μ M amsacrine, a concentration within the reported therapeutic range (3–18 μ M). Several AP parameters were significantly altered by 10 μ M amsacrine such as $V + \max$, AP peak, APA, Plateau₂₀ amplitude, and Plateau₅₀ amplitude values even if washout values were considered together with BTY and amsacrine values. The reduction in $V + \max$ was $58.2 \pm 9.4\%$ (Fig. 4B). AP peak was reduced by $55.5 \pm 10.0\%$ (Fig. 4D), and similarly to the experiments done with 1 μ M amsacrine, here we artificially set this value in the presence of 10 μ M amsacrine to 1 mV in three cells. 10 μ M amsacrine reduced APA by $16.3 \pm 3.3\%$ (Fig. 4C), Plateau₂₀ amplitude by $8.3 \pm 1.7\%$ (Fig. 5C), and Plateau₅₀ amplitude by $14.4 \pm 2.2\%$ (Fig. 5D). 10 μ M amsacrine-induced changes were mostly reversible indicated by the lack of significant difference between BTY and washout (Figs. 4, 5).

There were some AP parameters where significant difference could only be obtained if we compared their values between BTY and amsacrine (and not considering washout) (Fig. 5). Just as with 1 μ M amsacrine, the maximal rates of phase 1 and 3 were reduced in the presence of 10 μ M amsacrine as well. V_{Ph1max} was reduced by $61.9 \pm 7.3\%$, while the reduction in case of $V - \max$ was $21.1 \pm 3.5\%$ (Fig. 5).

The other determined AP parameters were not altered by 10 μ M amsacrine (Table 2).

Comparing the effects of 1 and 10 μ M amsacrine, % reduction values induced by amsacrine were only statistically significant with $V - \max$, Plateau₂₀ and Plateau₅₀ amplitudes, where the effect of 10 μ M amsacrine was higher (Table 3).

Table 1

AP parameters recorded before, in the presence of 1 μ M amsacrine, and after its washout.

AP parameter (unit)	BTY (control)	1 μ M amsacrine	Washout	Statistical test(s)	p value (s) between BTY and AMSA
APA (mV)	120.4 $\pm$ 1.3	102.2 $\pm$ 4.8	111.9 $\pm$ 2.3	K-W followed by Dunn post-hoc with Bonferroni correction	0.004
AP peak (mV)	34.3 $\pm$ 1.4	16.6 $\pm$ 4.9	24.8 $\pm$ 3.6	one-way ANOVA followed by Tukey post-hoc	0.004
APD ₂₀ (ms)	114.3 $\pm$ 13.3	147.5 $\pm$ 19.7	122.3 $\pm$ 21.4	one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.385/ 0.013
APD ₅₀ (ms)	224.5 $\pm$ 20.2	240.8 $\pm$ 20.2	228.8 $\pm$ 26.2	one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.85/ 0.134
APD ₇₅ (ms)	261.1 $\pm$ 20.8	274.2 $\pm$ 20.2	267.1 $\pm$ 25.5	K-W followed by Dunn post-hoc with Bonferroni correction/ Wilcoxon signed-rank test	1.0/ 0.232
APD ₉₀ (ms)	272.1 $\pm$ 20.9	283.6 $\pm$ 20.2	277.9 $\pm$ 25.2	K-W followed by Dunn post-hoc with Bonferroni correction/ Wilcoxon signed-rank test	1.0/ 0.557
APD ₅₀ /APD ₉₀	0.82 $\pm$ 0.02	0.84 $\pm$ 0.02	0.81 $\pm$ 0.02	one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.623/ 0.108
Plateau20 Amp (mV)	104.9 $\pm$ 2.3	101.2 $\pm$ 2.7	101.4 $\pm$ 2.9	K-W followed by Dunn post-hoc with Bonferroni correction/ Wilcoxon signed-rank test	0.473/ 0.037
Plateau50 Amp (mV)	89.1 $\pm$ 2.6	83.7 $\pm$ 3.1	84.4 $\pm$ 3.0	one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.623/ 0.015
RMP (mV)	-86.1 $\pm$ 1.2	-85.6 $\pm$ 2.0	-87.1 $\pm$ 2.4	one-way ANOVA followed by Tukey post-	0.982/ 0.626

Table 1 (continued)

AP parameter (unit)	BTY (control)	1 μ M amsacrine	Washout	Statistical test(s)	p value (s) between BTY and AMSA
SV (ms)	4.8 $\pm$ 0.7	4.7 $\pm$ 0.6	4.8 $\pm$ 0.9	hoc/ Paired <i>t</i> -test one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.993/ 0.842
V + max (V/s)	260.5 $\pm$ 24.9	149.2 $\pm$ 44.1	171.0 $\pm$ 31.9	K-W followed by Dunn post-hoc with Bonferroni correction	0.023
V _{Ph1} max (V/s)	-11.7 $\pm$ 2.5	-5.9 $\pm$ 2.0	-8.0 $\pm$ 2.8	K-W followed by Dunn post-hoc with Bonferroni correction/ Wilcoxon signed-rank test	0.151/ 0.002
V-max (V/s)	-1.81 $\pm$ 0.12	-1.71 $\pm$ 0.12	-1.70 $\pm$ 0.14	one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.826/ 0.04

Data are mean $\pm$ SEM from ten cells obtained from five animals except in washout (8 cells from 4 dogs). Explanation of abbreviations is given in the list of abbreviations. Italic fonts indicate non-normal distribution.

3.3. Amsacrine-sensitive current profile with AP voltage clamp (APVC)

In APVC experiments, each cell was stimulated with a previously recorded „canonic” action potential (Fig. 6A) to be able to compare the amsacrine-sensitive current among the tested cells with various AP shapes. Representative traces are shown on Fig. 6 and properties of the components of amsacrine-sensitive currents are summarized in Tables 4 and 5.

The high variation in the presence of the early outward component (present in 6 out of 12 cells with 1 and 4 out of 10 cells in 10 μ M amsacrine) is most likely due to the origin of the examined cells as I_{to} density is highly dependent on the transmural origin and increasing from endo to epi direction [26].

Comparing the effects of 1 and 10 μ M amsacrine-sensitive currents, no statistical difference could be observed in any parameters. The tendency for a difference was greatest in case of the early outward peak density values (0.99 ± 0.34 and 2.93 ± 1.04 pA/pF in 1 and 10 μ M amsacrine, respectively, $p = 0.129$).

3.4. Amsacrine-induced change of L-type calcium current

Based on the effects of amsacrine on the AP parameters and the properties of the amsacrine-sensitive currents amsacrine seems to inhibit at least the early and late sodium and the transient outward potassium current on top of the expected I_{Kr} inhibition. The inward current seen on amsacrine-sensitive currents during the plateau phase can also be due to the inhibition of $I_{Ca,L}$ and not just late sodium current inhibition. Therefore we measured the effect of amsacrine on $I_{Ca,L}$. Amsacrine, perfused at 10 μ M concentration significantly reduced the density of $I_{Ca,L}$ from -5.89 ± 0.64 to -4.61 ± 0.58 pA/pF ($22.2 \pm 2.7\%$ reduction) but only if washout (-5.66 ± 0.64 pA/pF) was not

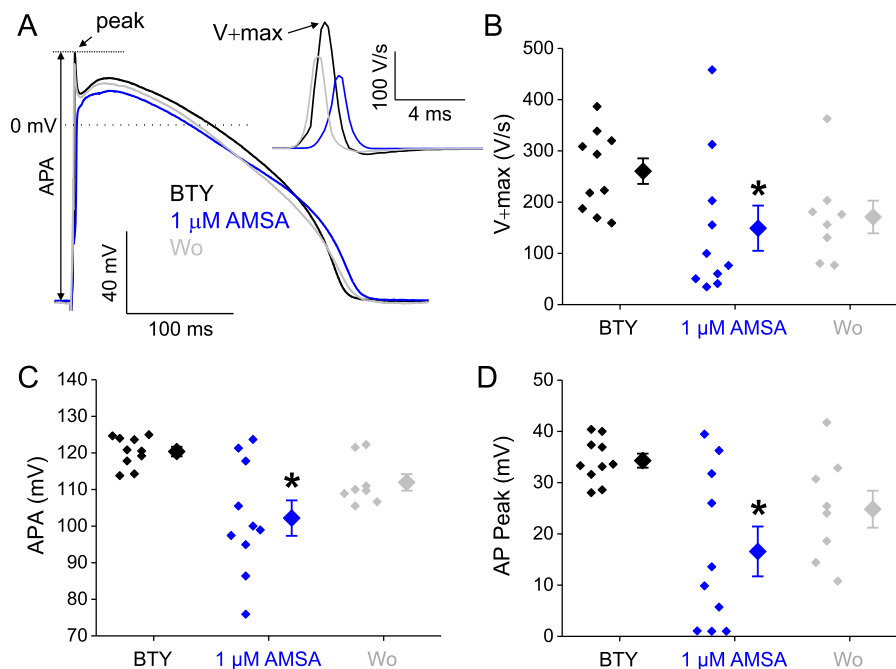


Fig. 2. 1 μ M amsacrine (AMSA) inhibits phase 0 of left ventricular cell AP. (A) Representative action potentials with their first time derivative between 13 and 25 ms (right insert). The black trace is the AP measured in BTY (control solution), the blue in the presence of 1 μ M amsacrine (AMSA), while the gray is after washout (Wo). (B–D) Diagrams showing AP parameters in control condition (BTY, black), in the presence of amsacrine (1 μ M AMSA, blue) and after washout of amsacrine (Wo, grey) where filled diamond symbols, large diamonds, and whiskers show the individual values, means, and $\pm$ SEM, respectively. Results were obtained from ten cells obtained from five animals except in washout (8 cells from 4 dogs). Asterisks show statistically significant difference from BTY among groups ($p < 0.05$).

considered and comparison was made only between 10 μ M amsacrine and control (Fig. 7B). $I_{Ca,L}$ activation was not influenced by amsacrine (the duration to the current peak from the start of depolarization was 3.02 ± 0.20 , 3.83 ± 0.55 , and 3.08 ± 0.36 ms in control, 10 μ M amsacrine, and in washout, respectively). Inactivation was judged by comparing the time constants of the double exponential fits of inactivation of the current traces. The time constant of the fast component was significantly higher in 10 μ M amsacrine but only if paired comparison was made between control (8.10 ± 0.49 ms) and amsacrine (9.53 ± 0.65 ms) and not considered the washout (10.09 ± 0.78 ms) (Fig. 7C). The time constant of the slowly inactivating component was not significantly affected by amsacrine (55.3 ± 3.0 , 58.0 ± 5.0 , and 69.0 ± 6.2 ms in control, amsacrine and washout, respectively). If we calculated the area under the calcium current traces which is equivalent to the carried charge density, its normalized value was significantly smaller in the presence of 10 μ M amsacrine (-74.6 ± 15.3 fC/pF) compared to control (-106.0 ± 15.7 fC/pF) (Fig. 7D). The normalized charge hardly increase after washout (-77.9 ± 12.7 fC/pF).

3.5. Amsacrine-induced change of transient outward potassium current

As mentioned before, the contour of amsacrine-sensitive currents (namely its early outward peak) as well as the reduction of V_{Ph1max} indicated a possible transient outward potassium current inhibition of amsacrine. To confirm this hypothesis, we measured the effect of 10 μ M amsacrine on I_{to} . Amsacrine significantly reduced the density of I_{to} from 14.7 ± 1.7 to 8.8 ± 1.1 pA/pF ($38.4 \pm 2.1\%$ reduction) (Fig. 8B). After washout of amsacrine I_{to} density was 12.7 ± 1.5 pA/pF. I_{to} activation was not influenced by amsacrine (the duration to the current peak from the start of depolarization was 2.02 ± 0.15 , 2.04 ± 0.27 , and 2.01 ± 0.17 ms in control, 10 μ M amsacrine, and in washout, respectively). Inactivation was judged by comparing the time constants of the double exponential fits of inactivation of the current traces. The time constant

of the fast component was significantly smaller in 10 μ M amsacrine (2.38 ± 0.24 ms) than in control (3.35 ± 0.49 ms) but this parameter after washout (2.87 ± 0.14 ms) was not different from either control or amsacrine values (Fig. 8C). The time constant of the slowly inactivating component was not significantly affected by amsacrine (12.6 ± 0.9 , 13.8 ± 1.9 , and 14.0 ± 1.0 ms in control, amsacrine and washout, respectively). Not only the current density, but also the normalized charge carried by the current was significantly smaller in the presence of 10 μ M amsacrine (53.2 ± 7.1 fC/pF) compared to both control (124.0 ± 16.5 fC/pF) and washout (100.6 ± 13.1 fC/pF) values (Fig. 8D).

3.6. Amsacrine blocks native I_{Kr}

As amsacrine-induced hERG blockade was described in detail before [14], but native I_{Kr} has not been studied earlier and therefore was tested in this study. Amsacrine induced a concentration-dependent reduction of I_{Kr} tail current with an IC_{50} of 151.6 ± 18.1 nM and a 1.208 ± 0.19 Hill coefficient (Fig. 9B).

3.7. Effect of amsacrine on expressed $Na_v1.5$ channels

Based on the results of action potentials (reduction of V_{+max}) amsacrine could potentially inhibit sodium current. As $Na_v1.5$ is responsible for cardiac sodium current [27,28] we tested the action of 10 μ M amsacrine on $Na_v1.5$ current expressed in CHO cells (Fig. 10). When applied extracellularly via the perfusion system, 10 μ M amsacrine caused a quick and reversible slight reduction of the whole-cell $Na_v1.5$ current ($RCF = 0.89 \pm 0.02$, $n = 4$, Fig. 10B) which was reversible upon washing with control solution. The rate of recovery from inactivation was significantly slowed by amsacrine ($n = 3$, $p = 0.018$) but was not reversible as assessed by fitting the fraction of recovered channels plotted as a function of interpulse interval (Fig. 10C). Time constant values were 7.97 ± 0.83 , 16.31 ± 0.81 , and 15.38 ± 2.20 ms in control,

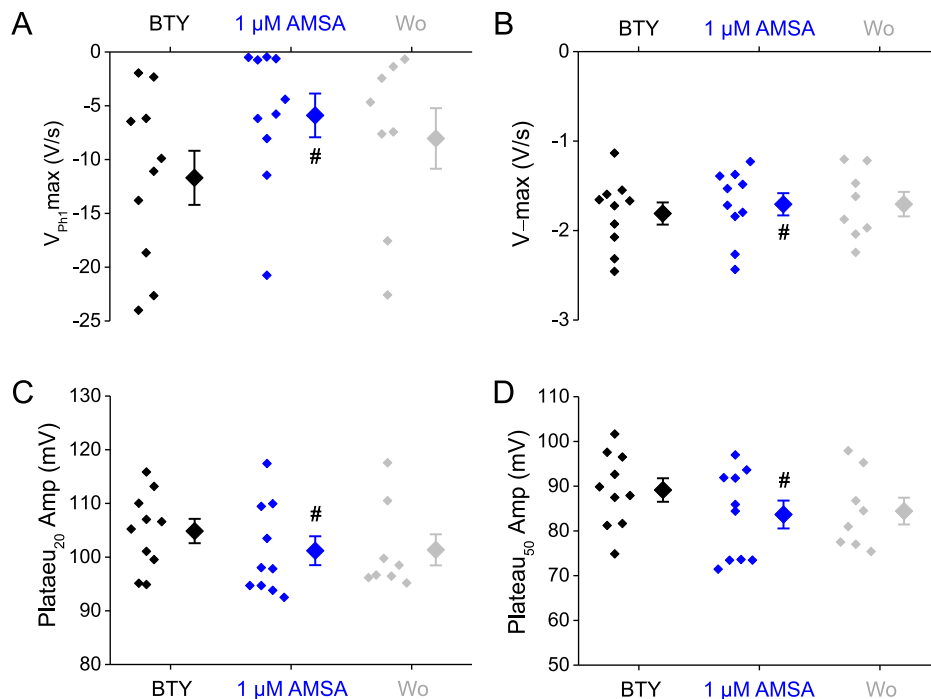


Fig. 3. Effects of 1 μ M amsacrine (AMSA) on further AP parameters. Diagrams showing AP parameters in control condition (BTY, black), in the presence of amsacrine (1 μ M AMSA, blue) and after washout of amsacrine (Wo, gray) where small filled diamonds, large diamonds, and whiskers show the individual values, means, and $\pm$ SEM, respectively. Values of V_{ph1max} (A), V_{-max} (B), plateau₂₀ amplitudes (difference between resting membrane potential and the membrane potential at 20% of APD₉₀, C), and plateau₅₀ amplitudes (difference between resting membrane potential and the membrane potential at 50% of APD₉₀, D) are shown. Results were obtained from ten cells obtained from five animals except in washout (8 cells from 4 dogs). Hashtags show statistically significant difference between BTY and AMSA ($p < 0.05$).

in 10 μ M amsacrine, and after washout, respectively. As a result, cumulative inactivation was enhanced, which was apparent by the faster decay of the current amplitude during high frequency pulsing (3.33 Hz, corresponding to a 200 bpm heart rate) and the lower remaining current fraction by the end of the 30 s pulsing period (Fig. 10D). Amsacrine significantly enhanced the extent of cumulative inactivation ($n = 4$, $p = 0.002$) (RCF values were 62.0 ± 4.7 , 10.0 ± 2.8 , and $39.2 \pm 7.8\%$ in control, in 10 μ M amsacrine, and after washout, respectively).

4. Discussion

To our best knowledge, this is the first report on the detailed characterization of amsacrine-induced action on ventricular cardiomyocytes. Even more importantly, we used canine myocytes as a model species, which is very similar to human heart in regard of cardiac electrophysiology [29,30]. The main finding of this study is that amsacrine significantly alters the configuration of the ventricular action potential without prolonging action potential duration, despite its known inhibitory effect on hERG channels. This apparent discrepancy can be explained by the simultaneous inhibition of multiple inward and outward ionic currents.

4.1. Effects of amsacrine on cardiac AP morphology

Although hERG is the pore-forming subunit of the I_{Kr} current [31], the native current might contain other regulatory subunits such as minK or MIRP [32,33]. The action of a compound might not be the same on native channels as on the expressed pore-forming subunit (containing no other regulatory proteins) as we have shown previously for the effect of TTX on calcium channels [34]. Therefore, we tested the action of 1 and 10 μ M amsacrine on the ventricular AP, where I_{Kr} is a major current determining late repolarization. We have first chosen 1 μ M

concentration as it is close to the IC₅₀ of amsacrine on hERG channels expressed in HEK cells [14].

Amsacrine induced marked changes in the early phases of the action potential. The substantial reduction in maximal upstroke velocity, action potential amplitude, and peak potential detected in both 1 and 10 μ M amsacrine (Figs. 2 and 4) strongly indicates inhibition of the fast sodium current (I_{Na}). This interpretation is supported by the prominent early inward component observed in the amsacrine-sensitive current under action potential voltage-clamp conditions (Fig. 6E, and discussed further later) as well as by the direct Nav1.5 reduction detected in the presence of amsacrine (Fig. 10).

The reduction in the maximal rate of phase 1 repolarization observed in both 1 and 10 μ M amsacrine (Figs. 3A and 5A) suggests suppression of the transient outward potassium current (I_{to}), which is consistent with the presence of an early outward component in the APVC recordings (Fig. 6) (discussed later) as well as with the results of direct I_{to} measurements (Fig. 8).

Furthermore, the decrease in plateau potentials (Figs. 3C-D and 5C-D) indicates amsacrine-induced inhibition of inward currents active during the plateau phase, most likely $I_{Ca,L}$ and $I_{Na,L}$. In theory, the reduction of plateau potential values can also be due to the increase of outward plateau currents. This possibility is unlikely as hERG channels and native I_{Kr} (Fig. 9) were reduced by amsacrine [14] and I_{K1} increase would lead to higher APD₅₀/APD₉₀ values as well as more negative values of V_{-max} and resting membrane potential. These were not detected, moreover, V_{-max} was reduced supporting I_{Kr} (and possibly some I_{K1}) inhibition (Figs. 3B and 5B). Plateau potential reduction can therefore be due to inward current inhibition in the form of $I_{Na,L}$ or $I_{Ca,L}$ blockade, the latter one was indeed detected in direct $I_{Ca,L}$ measurements (Fig. 7) whereas Nav1.5 current (mainly responsible for I_{Na} and $I_{Na,L}$) was also reduced by amsacrine (Fig. 10).

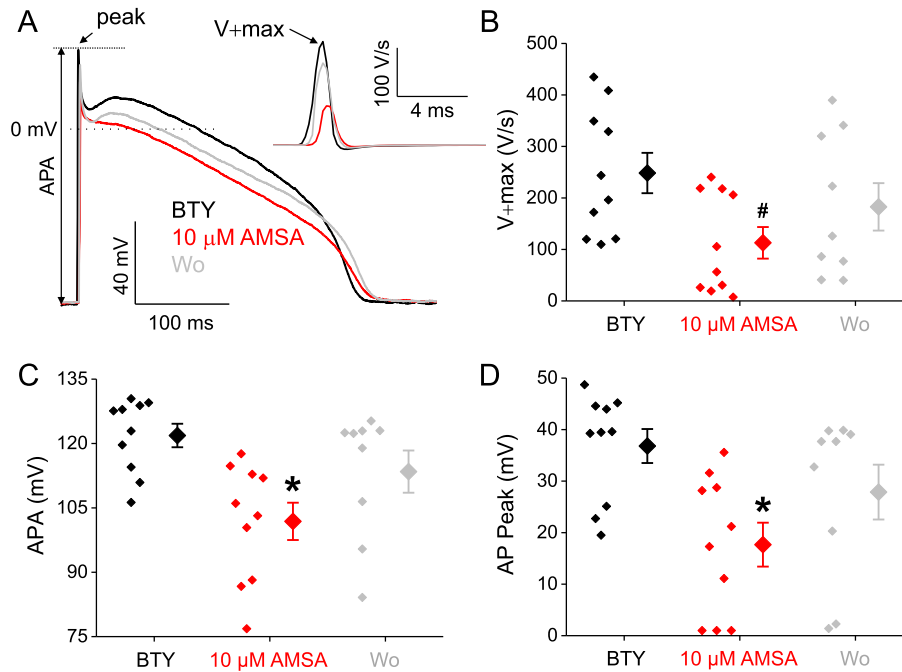


Fig. 4. 10 μM amsacrine (AMSA) inhibits phase 0 of left ventricular cell AP. (A) Representative action potentials with their first time derivative between 13 and 25 ms (right insert). The black trace is the AP measured in BTY (control solution), the red in the presence of 10 μM amsacrine (AMSA), while the gray is after washout (Wo). (B-D) Diagrams showing AP parameters in control condition (BTY, black), in the presence of amsacrine (10 μM AMSA, red) and after washout of amsacrine (Wo, grey) where filled diamond symbols, large diamonds, and whiskers show the individual values, means, and $\pm$ SEM, respectively. Results were obtained from ten cells obtained from four animals except in washout (9 cells from 4 dogs). Asterisks show statistically significant difference from BTY among groups ($p < 0.05$).

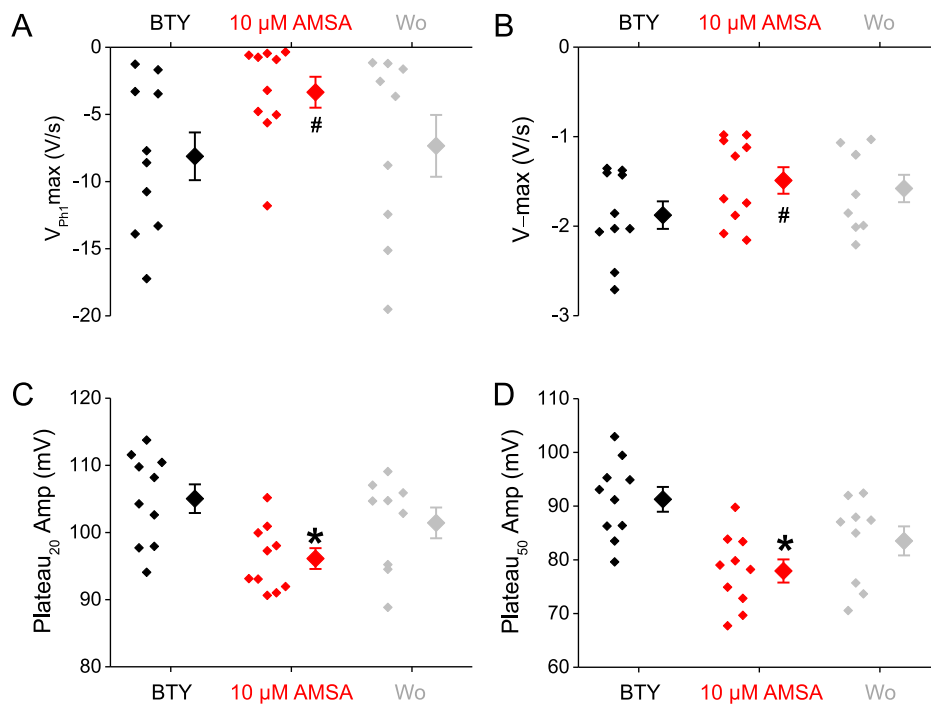


Fig. 5. 10 μM amsacrine (AMSA) reduced the plateau phase of left ventricular myocyte AP. Diagrams showing AP parameters in control condition (BTY, black), in the presence of amsacrine (10 μM AMSA, red) and after washout of amsacrine (Wo, gray) where small filled diamonds, large diamonds, and whiskers show the individual values, means, and $\pm$ SEM, respectively. Values of V_{ph1}max (A), V_{-max} (B), plateau₂₀ amplitudes (C), and plateau₅₀ amplitudes (D) are indicated. Results were obtained from ten cells obtained from four animals except in washout (9 cells from 4 dogs). Asterisks and hashtags show statistically significant difference from BTY among groups ($p < 0.05$) and between BTY and AMSA ($p < 0.05$), respectively.

Table 2

AP parameters recorded before, in the presence of 10 μ M amsacrine, and after its washout.

AP parameter (unit)	BTY (control)	10 μ M amsacrine	Washout	Statistical test(s)	p value (s) between BTY and AMSA
APA (mV)	121.9 $\pm$ 2.7	101.9 $\pm$ 4.3	109.7 $\pm$ 5.8	K-W followed by Dunn post-hoc with Bonferroni correction	0.005
AP peak (mV)	36.6 $\pm$ 3.3	17.7 $\pm$ 4.3	26.5 $\pm$ 4.9	K-W followed by Dunn post-hoc with Bonferroni correction	0.005
APD ₂₀ (ms)	120.8 $\pm$ 20.8	158.1 $\pm$ 41.6	121.8 $\pm$ 22.7	one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.652/ 0.261
APD ₅₀ (ms)	246.7 $\pm$ 26.6	255.0 $\pm$ 39.7	246.9 $\pm$ 28.8	one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.982/ 0.699
APD ₇₅ (ms)	285.1 $\pm$ 28.3	292.2 $\pm$ 39.4	296.7 $\pm$ 30.0	one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.987/ 0.679
APD ₉₀ (ms)	295.9 $\pm$ 28.8	303.0 $\pm$ 39.5	311.4 $\pm$ 29.5	one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.987/ 0.668
APD ₅₀ /APD ₉₀	0.83 $\pm$ 0.02	0.82 $\pm$ 0.03	0.78 $\pm$ 0.03	K-W followed by Dunn post-hoc with Bonferroni correction/ Wilcoxon signed-rank test	1.0/ 0.695
Plateau ₂₀ Amp (mV)	105.0 $\pm$ 2.1	96.1 $\pm$ 1.6	97.6 $\pm$ 4.3	K-W followed by Dunn post-hoc with Bonferroni correction	0.023
Plateau ₅₀ Amp (mV)	91.3 $\pm$ 2.3	77.9 $\pm$ 2.2	77.9 $\pm$ 2.2	K-W followed by Dunn post-hoc with Bonferroni correction	0.004
RMP (mV)	-85.0 $\pm$ 1.0	-84.2 $\pm$ 1.1	-83.2 $\pm$ 2.5	K-W followed by Dunn post-hoc with Bonferroni correction/ Wilcoxon signed-rank test	1.0/ 0.232
SV (ms)	4.3 $\pm$ 0.7	3.0 $\pm$ 0.5	5.2 $\pm$ 0.8	one-way ANOVA followed by	0.415/ 0.097

Table 2 (continued)

AP parameter (unit)	BTY (control)	10 μ M amsacrine	Washout	Statistical test(s)	p value (s) between BTY and AMSA
V + max (V/s)	248.4 $\pm$ 39.1	112.8 $\pm$ 30.7	182.7 $\pm$ 46.0	Tukey post-hoc/ Paired <i>t</i> -test K-W followed by Dunn post-hoc with Bonferroni correction/ Wilcoxon signed-rank test	0.051/ 0.002
V _{ph1} max (V/s)	-8.1 $\pm$ 1.8	-3.3 $\pm$ 1.1	-7.3 $\pm$ 2.3	K-W followed by Dunn post-hoc with Bonferroni correction/ Wilcoxon signed-rank test	0.087/ 0.002
V-max (V/s)	-1.88 $\pm$ 0.15	-1.49 $\pm$ 0.15	-1.58 $\pm$ 0.15	one-way ANOVA followed by Tukey post-hoc/ Paired <i>t</i> -test	0.224/ < 0.001

Data are mean $\pm$ SEM from ten cells obtained from four animals. Explanation of abbreviations is in the list of abbreviations. Italic fonts indicate non-normal distribution.

4.2. Lack of action potential prolongation despite I_{Kr} inhibition

Previous studies demonstrated that amsacrine inhibits hERG channels and we have confirmed that on native I_{Kr} current (Fig. 9), as well as QT prolongation in some human patients [4–6] as well as in certain animal models such as in dogs [13]. Despite this, we did not observe significant prolongation of action potential in our experiments at either amsacrine concentration. This finding is central to the interpretation of our results.

The most plausible explanation is that the reduction of inward plateau currents ($I_{Na,L}$ and $I_{Ca,L}$), together with inhibition of I_{Na} , counterbalances the effect of I_{Kr} inhibition on repolarization. Such simultaneous modulation of multiple ionic currents can preserve overall action potential duration despite significant changes in individual current components. This highlights the importance of considering integrated electrophysiological effects rather than single-channel data when assessing proarrhythmic risk.

4.3. Insights from action potential voltage-clamp experiments

Based on our current-clamp results, amsacrine could influence several ion currents including I_{to} , I_{K1} , late I_{Na} , and $I_{Ca,L}$ on top of I_{Kr} . We have used the APVC method which has the advantage of simultaneously recording all ion currents which are influenced (either reduced or increased) by the tested compound [35], in this case amsacrine. To interpret these results of amsacrine-sensitive current traces with APVC results it must be noted at first that amsacrine-sensitive current was determined by subtracting the current recorded in the presence of amsacrine (after the development of its actions) from that recorded right before the application of amsacrine, and therefore represents the sum of all currents modified by amsacrine. Secondly, a drug-sensitive current appears outward not only when an outward current is blocked, but also when an inward current is enhanced during the AP, like in case of

Table 3

AP parameter changes in the two tested amsacrine concentrations.

AP parameter change (unit)	1 μ M amsacrine-induced effect	10 μ M amsacrine-induced effect	Statistical test	p value between BTY and AMSA
APA (%)	-15.2 ± 3.6	-16.3 ± 3.3	Unpaired <i>t</i> -test	0.827
AP peak (%)	-54.2 ± 12.4	-55.5 ± 10.0	Unpaired <i>t</i> -test	0.936
APD ₂₀ (%)	$+30.6 \pm 9.7$	$+29.4 \pm 20.8$	Unpaired <i>t</i> -test	0.959
APD ₅₀ (%)	$+8.3 \pm 4.6$	$+0.3 \pm 7.0$	Mann-Whitney	0.123
APD ₇₅ (%)	$+5.9 \pm 4.8$	$+0.0 \pm 4.9$	Mann-Whitney	0.971
APD ₉₀ (%)	$+5.2 \pm 4.7$	$+0.1 \pm 4.6$	Mann-Whitney	1.000
APD ₅₀ /APD ₉₀ (%)	$+3.1 \pm 1.7$	-0.6 ± 2.4	Unpaired <i>t</i> -test	0.212
Plateau ₂₀ AMP (%)	-3.5 ± 1.4	-8.3 ± 1.7	Unpaired <i>t</i> -test	0.044
Plateau ₅₀ AMP (%)	-6.1 ± 2.1	-14.4 ± 2.2	Unpaired <i>t</i> -test	0.014
RMP (%)	-0.6 ± 1.8	-1.0 ± 0.7	Unpaired <i>t</i> -test	0.772
SV (%)	$+4.2 \pm 10.5$	-16.5 ± 18.2	Mann-Whitney	0.105
V + max (%)	-45.9 ± 11.9	-58.2 ± 9.4	Unpaired <i>t</i> -test	0.431
V _{Ph1} max (%)	-55.8 ± 9.2	-61.9 ± 7.3	Unpaired <i>t</i> -test	0.606
V-max (%)	-5.5 ± 2.6	-21.1 ± 3.5	Mann-Whitney	0.001

Data are mean $\pm$ SEM from ten cells obtained from five animals and ten cells obtained from four animals in case of 1 and 10 μ M amsacrine, respectively. Explanation of abbreviations is in the list of abbreviations. Italic fonts indicate non-normal distribution.

ABT-333 [19]. Thirdly, knowing the characteristic profile/fingerprint of the ion currents during the course of the AP [23,35–37] allows us to identify the currents modified by amsacrine.

The action potential voltage-clamp experiments revealed a composite amsacrine-sensitive current with several distinct components. The early transient inward peak is consistent with inhibition of I_{Na} , whereas the subsequent outward component likely reflects suppression of I_{to} . During the plateau phase, the mid-plateau inward current component suggests inhibition of inward currents such as $I_{Ca,L}$ and/or $I_{Na,L}$ [23]. Based on the average size of mid-plateau values of $I_{Na,L}$ (approximately -0.2 pA/pF recorded as 1 μ M GS-967-sensitive current) and $I_{Ca,L}$ (approximately -0.4 pA/pF recorded as 1 μ M nisoldipine-sensitive current) it is likely that amsacrine can inhibit both of these currents. Amsacrine-induced $I_{Na,L}$ inhibition is very likely as amsacrine reduced those parameters of the AP (APA, AP peak, and V+max) which suggests inhibition of the early sodium current (I_{Na}) (Figs. 2 and 4). The main determinant (pore forming subunit) of I_{Na} is Nav1.5 which can also be involved in $I_{Na,L}$ together with Nav1.8 [38]. Therefore, it is highly likely that amsacrine inhibits both the early and the late sodium current. We have indeed detected a slight but significant and rate dependent reduction in Nav1.5 (Fig. 10).

If one suppose an amsacrine-induced $I_{Ca,L}$ inhibition, it would likely

Table 4

Characteristics of 1 μ M amsacrine-sensitive current recorded with APVC condition.

total studied cells (12/6)	n/N	density	position relative to canonic AP
first inward component	8/4	-9.5 ± 3.5 pA/pF	0.89 ± 0.26 ms after its peak
early outward component	6/4	0.99 ± 0.34 pA/pF	5.71 ± 2.97 ms after its peak, 5.53 ± 2.97 ms after its point of V _{Ph1} max, 5.14 ± 2.97 before its point of the incisura at half-duration of its APD ₉₀
plateau current	12/6	-0.67 ± 0.19 pA/pF	161.7 ± 4.2 ms after its peak
zero current	10/6		
late outward component	10/6	0.24 ± 0.05 pA/pF	186.8 ± 3.6 ms after its peak, ≈ 3 ms before its point of V-max

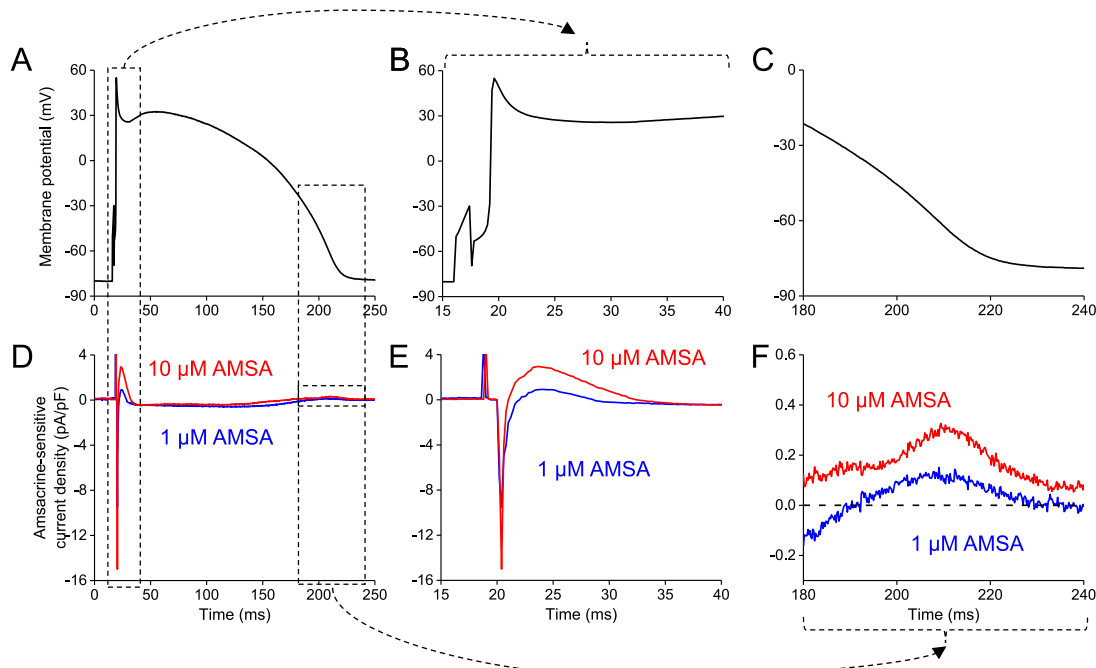


Fig. 6. Amsacrine-sensitive current in APVC condition.(A) The canonic action potential used as stimulus command with its enlarged parts indicated with dashed rectangles (B, C). Representative 1 μ M amsacrine-sensitive current (blue) and of 10 μ M amsacrine-sensitive current (red) (D) and their enlarged parts (E-F).

Table 5

Characteristics of 10 μ M amsacrine-sensitive current recorded with APVC condition.

total studied cells (10/5)	n/N	density	position relative to canonic AP
first inward component	10/5	-15.4 ± 3.6 pA/pF	0.84 ± 0.15 ms after its peak
early outward component	4/4	2.93 ± 1.04 pA/pF	3.34 ± 0.84 ms after its peak, 3.16 ± 0.84 ms after its point of V_{ph1max} , 7.51 ± 0.84 ms before its point of the incisura
plateau current	10/5	-0.51 ± 0.11 pA/pF	at half-duration of its APD ₉₀
zero current	9/5		158.4 ± 8.7 ms after its peak
late outward component	9/5	0.36 ± 0.09 pA/pF	181.2 ± 6.6 ms after its peak, ≈ 8.5 ms before its point of V-max

cause negative inotropic effect, which was not assessed in the current study but it is in good agreement with previous results obtained in both rabbits [9] and rats [11]. Indeed, we have shown that amsacrine can directly reduce $I_{Ca,L}$ as confirmed with conventional voltage clamp recordings where a partially reversible and approximately 22% size inhibition was seen (Fig. 7).

The early outward component of the amsacrine-sensitive current can be due to the amsacrine induced inhibition of I_{to} which is also supported by the reduction of V_{ph1max} parameter of the AP (Figs. 3A and 5A) as well as by direct current recordings (Fig. 8). The position of the early outward peak, if detected, was always after the first inward peak and was detected between the point of V_{ph1max} and the incisura of the canonic AP.

Finally, the late outward component observed during terminal repolarization is compatible with I_{Kr} inhibition. That component was only tended to be larger and appearing earlier in 10 μ M amsacrine (0.36 pA/pF and 8.5 ms before the position of V-max) compared to that of 1 μ M amsacrine (0.24 pA/pF and 3 ms before the position of V-max) (Fig. 6F). In case of our previous results when I_{Kr} was recorded as E-4031-sensitive current using either own [36] or canonic APs [23] during APVC recordings its peak of approximately 0.6 and 0.5 pA/pF was present 7 and 11 ms before the position of V-max, respectively. In those measurements 1 μ M E-4031 was applied which results in a nearly complete block of I_{Kr} . In hERG channels expressed in HEK cells 1 and 10 μ M amsacrine led to an approximate 80 and 95% inhibition [14]. Based on those findings and also the results of this study (approximately 90 and 95% I_{Kr} reduction by 1 and 10 μ M amsacrine, respectively) the late outward peaks of the amsacrine-sensitive currents detected in the current study should be larger. But as amsacrine-sensitive currents possessed an inward mid-plateau current which was not present in the E-4031-sensitive current it is likely that this inward current can reduce the late peak values. This inward component can also lead to the later appearance of the late peak in 1 μ M amsacrine.

Due to overlap in the time course of multiple ionic currents (I_{to} , $I_{Ca,L}$, and to a smaller extent also I_{Na}), precise quantification of individual current contributions is not possible using APVC alone. This can be observed in some of our previous publications where two local anesthetic reduced both I_{to} and $I_{Ca,L}$ and even if I_{to} inhibition was present as detected from conventional voltage clamp recordings that was not detectable with APVC due to overlap with $I_{Ca,L}$ inhibition [39,40]. Nevertheless, the temporal profile of the amsacrine-sensitive current strongly supports a multi-channel blocking effect which was confirmed by direct current recordings.

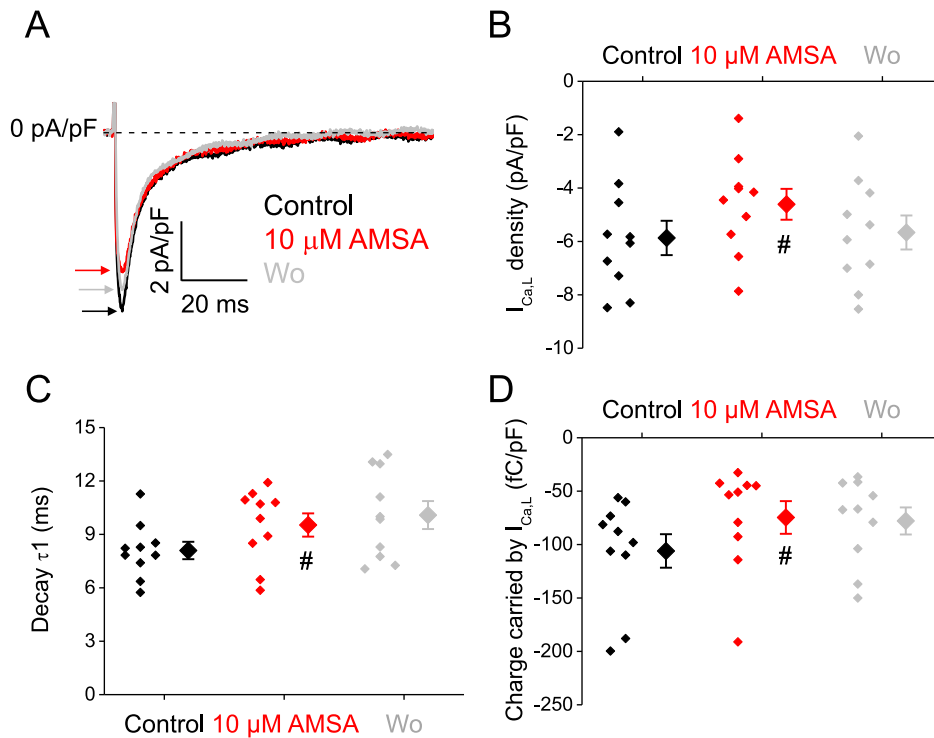


Fig. 7. Effects of 10 μ M amsacrine (AMSA) on L-type calcium current. (A) Representative traces of L-type calcium current normalized to cell capacitance with arrows indicating peaks. Diagrams showing $I_{Ca,L}$ density values (B), the time constant values of the fast inactivation component (C) and the normalized total charge carried by $I_{Ca,L}$ (D) in control condition (Control, black), in the presence of amsacrine (10 μ M AMSA, red) and after washout of amsacrine (Wo, grey) where filled diamond symbols, large diamonds, and whiskers show the individual values, means, and $\pm$ SEM, respectively. Results were obtained from ten cells obtained from three animals. Hash tags show statistically significant difference between Control and AMSA ($p < 0.05$).

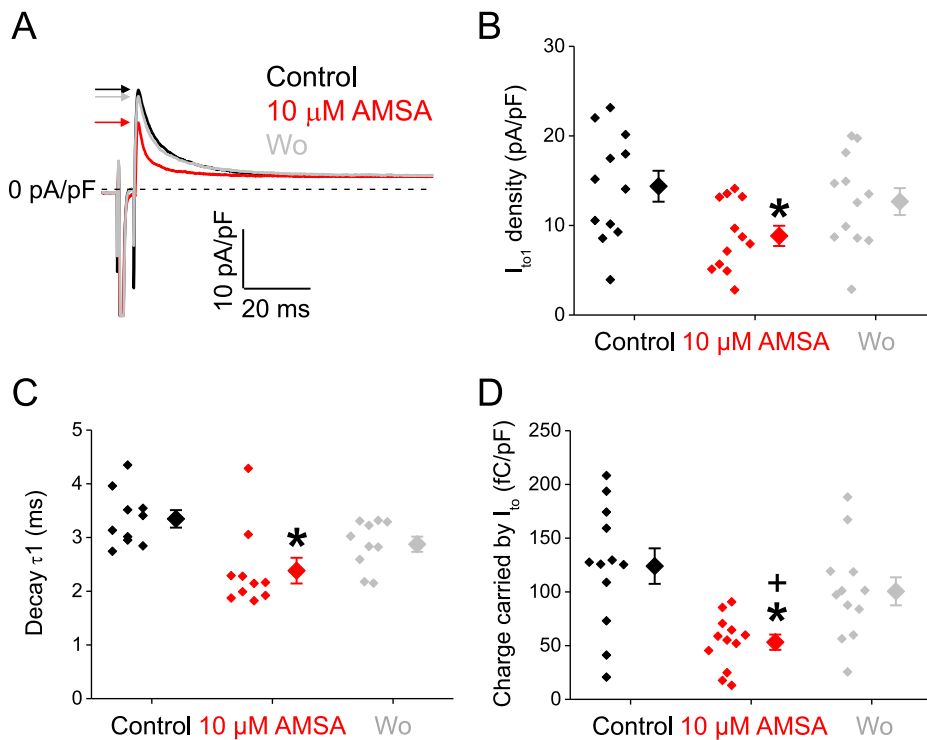


Fig. 8. Effects of 10 μM amsacrine (AMSA) on transient outward potassium current. (A) Representative traces of I_{to} normalized to cell capacitance with arrows indicating peaks. Diagrams showing I_{to} density values (B), the time constant values of the fast inactivation component (C) and the normalized total charge carried by I_{to} (D) in control condition (Control, black), in the presence of amsacrine (10 μM AMSA, red) and after washout of amsacrine (Wo, grey) where filled diamond symbols, large diamonds, and whiskers show the individual values, means, and $\pm$ SEM, respectively. Results were obtained from twelve cells obtained from three animals. Asterisks and + sign show statistically significant ($p < 0.05$) difference from BTY and from washout, respectively.

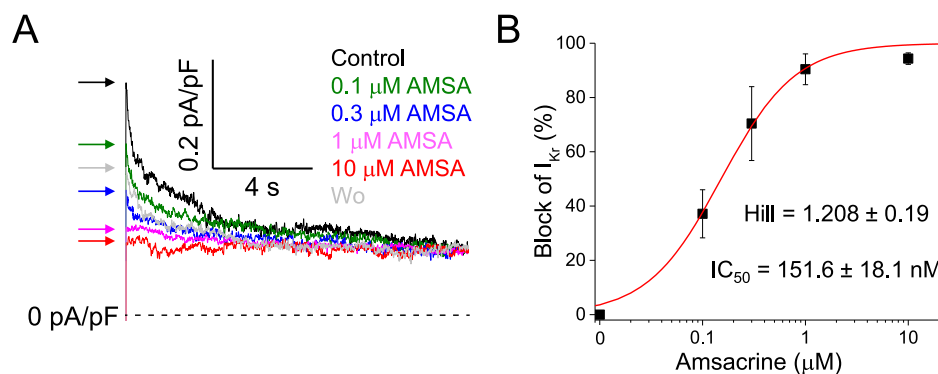


Fig. 9. Effects of 10 μM amsacrine (AMSA) on fast component of delayed rectifier potassium current. (A) Representative traces of I_{Kr} normalized to cell capacitance with arrows indicating peaks in control condition (Control, black), in the presence of various amsacrine concentrations (color coded) and after washout of amsacrine (Wo, grey). (B) Concentration-response curve of amsacrine induced I_{Kr} inhibition. Symbols and whiskers show means $\pm$ SEM values, red line is the fit. Results were obtained from three cells obtained from two animals.

4.4. Amsacrine-induced actions recorded by direct current measurements

To circumvent the previously mentioned temporal overlap of potential amsacrine-induced I_{to} and $I_{\text{Ca,L}}$ inhibition in APVC recordings we separated native I_{to} and $I_{\text{Ca,L}}$ and directly recorded the effect of 10 μM amsacrine on those (Figs. 7 and 8). The results of these measurements proved and nicely supported the effects of amsacrine not only on the respective AP parameters (reduction of V_{ph1max} and Plateau amplitudes) but also on the various parts of amsacrine-sensitive current in APVC recordings (appearance of an early outward peak and an inward plateau current). Both I_{to} and $I_{\text{Ca,L}}$ was significantly reduced in the

presence of 10 μM amsacrine by approximately 38 and 22%, respectively. The latter one was however only statistically significant if a paired comparison was performed between control and amsacrine. The faster inactivation of I_{to} (reduction of the fast inactivation time constant τ_1) could also contribute to the smaller overall carried charge and lead therefore to the large, more than 60% reduction of V_{ph1max} . Plateau amplitude reduction in 10 μM amsacrine was smaller (8 and 14% for plateau₂₀ and plateau₅₀) than that of V_{ph1max} which can be due to the much smaller amsacrine-induced $I_{\text{Ca,L}}$ reduction together with the lack of acceleration of inactivation. Indeed, the decay τ_1 was actually bigger indicating a small slowing of inactivation which can act against the

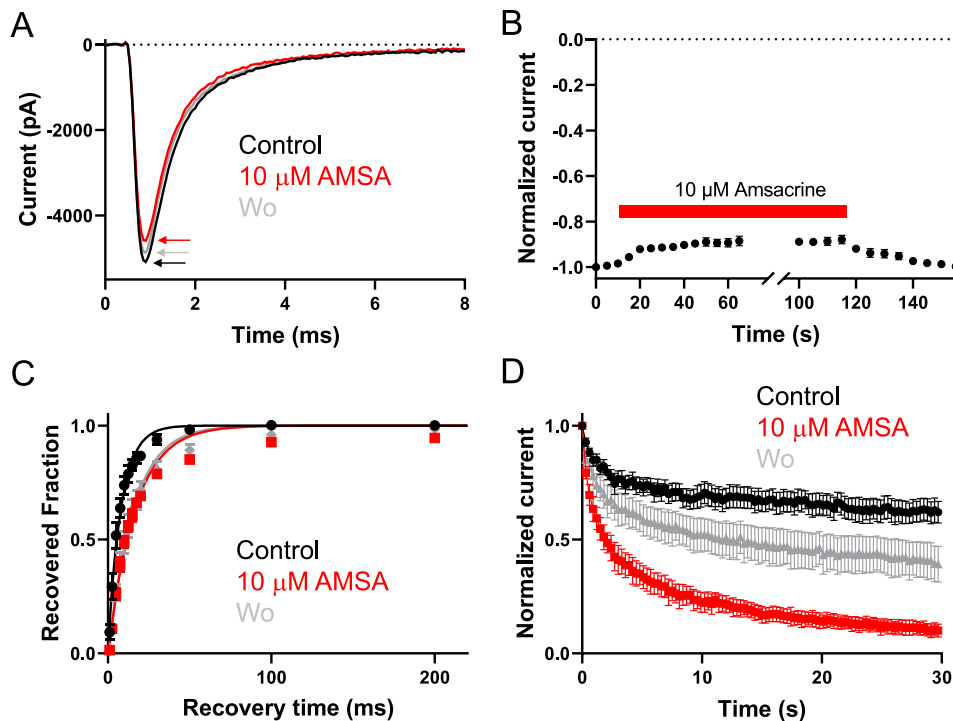


Fig. 10. Effects of 10 μM amsacrine (AMSA) on $\text{Na}_V1.5$ current. (A) Representative current traces with arrows indicating peaks. (B) Time course of amsacrine affect indicated the average $\pm$ SEM of normalized peak $\text{Na}_V1.5$ currents recorded in 4 cells. (C) Recovery from inactivation shown as the fraction of recovered channels as a function of recovery time (interpulse interval) in control condition (Control, black), in the presence of amsacrine (10 μM AMSA, red) and after washout of amsacrine (Wo, grey) ($n = 4$ in each case). (D) Cumulative inactivation as assessed by measuring the change in current amplitudes at a 3.33 Hz pulsing rate. Current amplitudes normalized to the amplitude of the first pulse are plotted against time ($n = 4$ in each case). Symbols indicate average $\pm$ SEM values.

carried charge reduction.

Moreover, $\text{Na}_V1.5$ current as well as native I_{Kr} was also reduced by 10 μM amsacrine. In case of $\text{Na}_V1.5$, amsacrine-induced inhibition was rather small (approximately 10% upon measured with 5-s-long interpulse interval pacing) (Fig. 10) but due to the significant reduction of channel recovery rate as a result cumulative inactivation the sodium current reduction was likely higher in our AP experiments due to the 1 Hz pacing. I_{Kr} reduction was confirmed with direct current measurements as well (Fig. 9). The IC_{50} value was approximately 152 nM and Hill coefficient was 1.21. This is in good agreement with the previously reported IC_{50} values of amsacrine obtained on expressed hERG channels in *Xenopus* oocytes (2 μM) and in HEK cells (209 nM) [14]. Hill coefficient was also identical in hERG and I_{Kr} recordings.

4.5. Lack of additional consequences of amsacrine application

Based on our results amsacrine indeed inhibits I_{Kr} (which was expected from its chemical structure and was proven by hERG channel experiments [14]), I_{to} (reduction of V_{ph1max} , early outward component of APVC-recorded amsacrine-sensitive current, as well as direct I_{to} recordings), $\text{I}_{\text{Ca,L}}$ (reduction of plateau₂₀ and plateau₅₀ values and direct current measurements), and $\text{Na}_V1.5$ current therefore most likely I_{Na} and $\text{I}_{\text{Na,L}}$ too. Drug-induced arrhythmia risk is often associated with action potential prolongation and increased spatial and temporal variability of repolarization. Although spatial heterogeneity of APD as not assessed here, temporal variability was calculated in the form of short term variability of repolarization (SV values in Tables 1 and 2) which was not influenced by either tested amsacrine concentrations. This suggests a relatively low proarrhythmic potential under the tested conditions.

Importantly, the absence of early afterdepolarizations further supports this conclusion. However, the pronounced inhibition of sodium

current may impair conduction, which could be clinically relevant, particularly in patients with pre-existing cardiac disease or electrolyte imbalance.

4.6. Limitations

Several limitations should be considered. First, the experiments were performed in isolated canine ventricular myocytes, which, although a well-established model for human electrophysiology, may not fully reproduce in vivo conditions. Second, individual ionic currents were not measured directly using conventional voltage-clamp protocols (except for I_{to} , I_{Kr} , and $\text{I}_{\text{Ca,L}}$), and conclusions are therefore based on indirect evidence from AP morphology and APVC recordings for the hypothesized I_{Na} and $\text{I}_{\text{Na,L}}$ inhibition despite the direct reduction of $\text{Na}_V1.5$ current. Third, only acute effects were studied; chronic exposure may lead to different electrophysiological and structural changes.

4.7. Clinical relevance and conclusions

The concentrations used in this study overlap with therapeutic plasma levels of amsacrine. Our results indicate that amsacrine exerts complex electrophysiological effects by simultaneously inhibiting multiple ion currents, including I_{Na} , I_{to} , $\text{I}_{\text{Ca,L}}$, $\text{I}_{\text{Na,L}}$, and I_{Kr} .

Despite I_{Kr} inhibition, the lack of action potential prolongation suggests a relatively low risk of torsadogenic effects under normal conditions. Moreover, amsacrine's arrhythmogenic potential is further reduced by placing the compound on the CredibleMeds website in 2018 with a note of conditional risk of TdP [8] resulting in careful monitoring of QT interval and plasma potassium levels during its application according to the recommendation.

However, given its multi-channel blocking profile, amsacrine may still pose a risk in patients with reduced repolarization reserve,

electrolyte disturbances, or underlying cardiac disease.

Overall, amsacrine should be considered a combined ion-channel modulator with a conditional risk of arrhythmia.

Abbreviations

4-AP	4-aminopyridine
AP	action potential
APA	action potential amplitude
APD	action potential duration
APD _{20, 50, 75, 90}	action potential duration at 20, 50, 75, and 90% of repolarization
APVC	action potential voltage clamp
BTY	bicarbonate buffer containing Tyrode solution
EAD	early afterdepolarization
HEK	human embryonic kidney
IC ₅₀	half maximal inhibitory concentration
I _{Ca,L}	L-type calcium current
I _{K1}	inward rectifier potassium current
I _{Kr}	rapid component of the delayed rectifier potassium current
I _{Ks}	slow component of the delayed rectifier potassium current
I _{Na}	early sodium current
I _{Na,L}	late sodium current
I _{to}	transient outward potassium current
LQTS2	long QT syndrome type 2
OSP	overshoot potential
Plateau ₂₀	amplitude membrane potential difference between RMP and the membrane potential value at 20% duration of APD ₉₀
Plateau ₅₀	amplitude membrane potential difference between RMP and the membrane potential value at 50% duration of APD ₉₀
RCF	remaining current fraction
RMP	resting membrane potential
SV	short-term variability of repolarization
TdP	torsade de pointes
V + max	maximal rate of phase 0 depolarization
V _{ph1} max	maximal rate of phase 1 repolarization
V–max	maximal rate of phase 3 repolarization

CRediT authorship contribution statement

József Óvári: Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Kovács Zsigmond:** Writing – review & editing, Methodology, Investigation, Formal analysis. **János Magyar:** Writing – review & editing. **Tamás Bányász:** Writing – review & editing. **Péter P. Nánási:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Balázs Horváth:** Writing – review & editing, Writing – original draft. **Zoltan Varga:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Norbert Szentandrassy:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

All authors confirmed no conflict of interest.

Acknowledgements

Financial support was provided by grants from the National Research Development and Innovation Office (NKFIH-K138090 to P.P.N.; NKFIH-K142764 to N.S.; NKFIH-K147301 to T.B.; EKÖP-25-3-II-DE-105 to J. O.). The work was supported by the University of Debrecen Program for Scientific Publication (NS, BH).

Funding sources had no involvement in preparation of the article; in study design; in the collection, analysis and interpretation of data; in

writing of the report; and in the decision to submit the article for publication.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2026.119825](https://doi.org/10.1016/j.biopha.2026.119825).

Data availability

Data will be made available on request.

References

- [1] J.F. Seymour, Induction of hypomagnesemia during Amsacrine treatment, *Am. J. Hematol.* 42 (3) (1993) 262–267.
- [2] T.J. Van Mouwerik, P.M. Caines, R. Ballentine, Amsacrine evaluation, *Drug. Intell. Clin. Pharm.* 21 (4) (1987) 330–334.
- [3] P.A. Cassileth, R.P. Gale, Amsacrine: a review, *Leuk. Res.* 10 (11) (1986) 1257–1265.
- [4] E. Shinar, Y. Hasin, Acute electrocardiographic changes induced by amsacrine, *Cancer Treat. Rep.* 68 (9) (1984) 1169–1172.
- [5] R.B. Weiss, D. Moquin, J.D. Adams, J.D. Griffin, H. Zimble, Electrocardiogram abnormalities induced by amsacrine, *Cancer Chemother. Pharmacol.* 10 (2) (1983) 133–134.
- [6] R.B. Weiss, A.J. Grillo-López, S. Marsoni, J.G. Posada, F. Hess, B.J. Ross, Amsacrine-associated cardiotoxicity: an analysis of 82 cases, *J. Clin. Oncol.* 4 (6) (1986) 918–928.
- [7] P. McLaughlin, P.G. Salvador, F. Cabanillas, S.S. Legha, Ventricular fibrillation following AMSA. Uncomplicated retreatment following correction of hypokalemia, *Cancer* 52 (3) (1983) 557–558.
- [8] <https://www.crediblemeds.org/blog/five-drugs-added-crediblemeds-qt-drugs-lists>.
- [9] N.D. Alessandro, N. Gebbia, M. Crescimanno, C. Flandina, G. Leto, F. M. Tumminello, L. Messina, Effects of amsacrine (m-AMSA), a new aminoacridine antitumor drug, on the rabbit heart, *Cancer Treat. Rep.* 67 (5) (1983) 467–474.
- [10] W.e al. <https://ntrl.ntis.gov/NTRL/dashboard/searchResults/titleDetail/PB81110421.xhtml>.
- [11] M.S. Merkin, E. Shinar, A. Shefer, M.S. Gotsman, Y. Hasin, The effects of m-AMSA on rat isolated heart, *Clin. Exp. Pharmacol. Physiol.* 12 (2) (1985) 131–138.
- [12] S.N. Kim, J.R. Watkins, U. Jayasekara, J.A. Anderson, J.E. Fitzgerald, F.A. de la Iglesia, Cardiotoxicity study of amsacrine in rats, *Toxicol. Appl. Pharmacol.* 77 (3) (1985) 369–373.
- [13] H.e al. <https://ntrl.ntis.gov/NTRL/dashboard/searchResults/titleDetail/PB264120.xhtml>.
- [14] D. Thomas, B.C. Hammerling, K. Wu, A.B. Wimmer, E.K. Ficker, G.E. Kirsch, M. C. Kochan, B.A. Wible, E.P. Scholz, E. Zitron, S. Kathöfer, V.A. Kreye, H.A. Katus, W. Schoels, C.A. Karle, J. Kiehn, Inhibition of cardiac HERG currents by the DNA topoisomerase II inhibitor amsacrine: mode of action, *Br. J. Pharmacol.* 142 (3) (2004) 485–494.
- [15] Z.M. Kovács, J. Óvári, J. Magyar, T. Bányász, P.P. Nánási, B. Horváth, G. Balogh, N. Szentandrassy, The methanesulfonamide group: bright and dark sides of hERG potassium channel inhibition, *Pharmaceuticals (Basel)* 19 (6) (2026).
- [16] M.E. Curran, I. Splawski, K.W. Timothy, G.M. Vincent, E.D. Green, M.T. Keating, A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome, *Cell* 80 (5) (1995) 795–803.
- [17] M.D. Perry, C.A. Ng, S.A. Mann, A. Sadrieh, M. Imtiaz, A.P. Hill, J.I. Vandenberg, Getting to the heart of hERG K(+) channel gating, *J. Physiol.* 593 (12) (2015) 2575–2585.
- [18] B. Christophe, Occurrence of early afterdepolarization under healthy or hypertrophic cardiomyopathy conditions in the human ventricular endocardial myocyte: In silico study using 109 torsadogenic or non-torsadogenic compounds, *Toxicol. Appl. Pharmacol.* 438 (2022) 115914.
- [19] Z.M. Kovács, J. Óvári, C. Dienes, J. Magyar, T. Bányász, P.P. Nánási, B. Horváth, A. Feher, Z. Varga, N. Szentandrassy, ABT-333 (Dasabuvir) increases action potential duration and provokes early afterdepolarizations in canine left ventricular cells via inhibition of I₁, *Pharmaceuticals (Basel)* 16 (4) (2023).
- [20] J. Magyar, N. Szentandrassy, T. Bányász, V. Kecskeméti, P.P. Nánási, Effects of norfluoxetine on the action potential and transmembrane ion currents in canine ventricular cardiomyocytes, *Naunyn Schmiede Arch. Pharmacol.* 370 (3) (2004) 203–210.
- [21] C. Dienes, T. Hézsó, D.Z. Kiss, D. Baranyai, Z.M. Kovács, L. Szabó, J. Magyar, T. Bányász, P.P. Nánási, B. Horváth, M. Gönczi, N. Szentandrassy, Electrophysiological effects of the transient receptor potential melastatin 4 channel inhibitor (4-Chloro-2-(2-chlorophenoxy)acetamido) benzoic acid (CBA) in canine left ventricular cardiomyocytes, *Int. J. Mol. Sci.* 22 (17) (2021).
- [22] N. Szentandrassy, K. Kistmász, B. Hegyi, B. Horváth, F. Ruzsnavszky, K. Váczi, J. Magyar, T. Bányász, A. Varró, P.P. Nánási, Contribution of ion currents to beat-to-beat variability of action potential duration in canine ventricular myocytes, *Pflug. Arch.* 467 (7) (2015) 1431–1443.
- [23] B. Horváth, D. Kiss, C. Dienes, T. Hézsó, Z. Kovács, N. Szentandrassy, J. Almássi, J. Magyar, T. Bányász, P.P. Nánási, Ion current profiles in canine ventricular

- myocytes obtained by the "onion peeling" technique, *J. Mol. Cell. Cardiol.* 158 (2021) 153–162.
- [24] K. Kistamás, N. Szentandrassy, B. Hegyi, F. Ruzsnavszky, K. Vácz, L. Bárándi, B. Horváth, A. Szebeni, J. Magyar, T. Bányász, V. Kecskeméti, P.P. Nánási, Effects of pioglitazone on cardiac ion currents and action potential morphology in canine ventricular myocytes, *Eur. J. Pharmacol.* 710 (1-3) (2013) 10–19.
- [25] J. Óvári, Z.M. Kovács, C. Dienes, J. Magyar, T. Bányász, P.P. Nánási, B. Horváth, G. J. Domingos, A. Feher, Z. Varga, N. Szentandrassy, Delavirdine modifies action potential configuration via inhibition of I, *Biomed. Pharmacother.* 186 (2025) 117994.
- [26] C. Antzelevitch, Molecular basis for the transmural distribution of the transient outward current, *J. Physiol.* 533 (Pt 1) (2001) 1.
- [27] N. Gaborit, S. Le Bouter, V. Szuts, A. Varro, D. Escande, S. Nattel, S. Demolombe, Regional and tissue specific transcript signatures of ion channel genes in the non-diseased human heart, *J. Physiol.* 582 (Pt 2) (2007) 675–693.
- [28] M.B. Rook, M.M. Evers, M.A. Vos, M.F. Bierhuizen, Biology of cardiac sodium channel Nav1.5 expression, *Cardiovasc. Res.* 93 (1) (2012) 12–23.
- [29] N. Szentandrassy, T. Banyasz, T. Biro, G. Szabo, B.I. Toth, J. Magyar, J. Lazar, A. Varro, L. Kovacs, P.P. Nanasi, Apico-basal inhomogeneity in distribution of ion channels in canine and human ventricular myocardium, *Cardiovasc. Res.* 65 (4) (2005) 851–860.
- [30] P.P. Nánási, B. Horváth, F. Tar, J. Almásky, N. Szentandrassy, N. Jost, I. Baczkó, T. Bányász, A. Varró, Canine myocytes represent a good model for human ventricular cells regarding their electrophysiological properties, *Pharmaceuticals (Basel)* 14 (8) (2021).
- [31] M.C. Sanguinetti, A. Zou, Molecular physiology of cardiac delayed rectifier K⁺ channels, *Heart Vessels Suppl.* 12 (1997) 170–172.
- [32] T.V. McDonald, Z. Yu, Z. Ming, E. Palma, M.B. Meyers, K.W. Wang, S.A. Goldstein, G.I. Fishman, A minK-HERG complex regulates the cardiac potassium current I(Kr), *Nature* 388 (6639) (1997) 289–292.
- [33] J.M. Nerbonne, Molecular basis of functional voltage-gated K⁺ channel diversity in the mammalian myocardium, *J. Physiol.* 525 Pt 2 (Pt 2) (2000) 285–298.
- [34] N. Szentandrassy, F. Papp, B. Hegyi, A. Bartok, Z. Krasznai, P.P. Nanasi, Tetrodotoxin blocks native cardiac L-type calcium channels but not CaV1.2 channels expressed in HEK cells, *J. Physiol. Pharmacol.* 64 (6) (2013) 807–810.
- [35] N. Szentandrassy, D. Nagy, F. Ruzsnavszky, G. Harmati, T. Bányász, J. Magyar, A. J. Szentmiklósi, P.P. Nánási, Powerful technique to test selectivity of agents acting on cardiac ion channels: the action potential voltage-clamp, *Curr. Med. Chem.* 18 (24) (2011) 3737–3756.
- [36] T. Bányász, J. Magyar, N. Szentandrassy, B. Horváth, P. Birinyi, J. Szentmiklósi, P. P. Nánási, Action potential clamp fingerprints of K⁺ currents in canine cardiomyocytes: their role in ventricular repolarization, *Acta Physiol. (Oxf.)* 190 (3) (2007) 189–198.
- [37] T. Bányász, L. Fülöp, J. Magyar, N. Szentandrassy, A. Varró, P.P. Nánási, Endocardial versus epicardial differences in L-type calcium current in canine ventricular myocytes studied by action potential voltage clamp, *Cardiovasc. Res.* 58 (1) (2003) 66–75.
- [38] B. Horváth, N. Szentandrassy, J. Almásky, C. Dienes, Z.M. Kovács, P.P. Nánási, T. Banyasz, Late sodium current of the heart: where do we stand and where are we going? *Pharmaceuticals (Basel)* 15 (2) (2022).
- [39] A. Szabó, N. Szentandrassy, P. Birinyi, B. Horváth, G. Szabó, T. Bányász, I. Márton, P.P. Nánási, J. Magyar, Effects of articaine on action potential characteristics and the underlying ion currents in canine ventricular myocytes, *Br. J. Anaesth.* 99 (5) (2007) 726–733.
- [40] A. Szabó, N. Szentandrassy, P. Birinyi, B. Horváth, G. Szabó, T. Bányász, I. Márton, J. Magyar, P.P. Nánási, Effects of ropivacaine on action potential configuration and ion currents in isolated canine ventricular cardiomyocytes, *Anesthesiology* 108 (4) (2008) 693–702.