Electrophysiologic and Antiarrhythmic Effects of AZD1305 in Canine Pulmonary Vein Sleeves

Serge Sicouri, Leif Carlsson, and Charles Antzelevitch

Masonic Medical Research Laboratory, Utica, NY, (S.S., C.A.) and AstraZeneca R&D, Mölndal, Sweden (L.C.)
Running title: Antiarrhythmic effects of AZD1305 in pulmonary vein sleeves

Address for editorial correspondence:
Serge Sicouri, MD
Masonic Medical Research Laboratory
2150 Bleecker Street
Utica, N.Y. 13501
Phone: (315)735-2217
FAX: (315)735-5648
E-mail: sicouris@mmrl.edu

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**ABBREVIATIONS:** ACh, Acetylcholine; AF, atrial fibrillation; Amio, amiodarone; ANOVA, analysis of variance; APA, action potential amplitude; APD50, action potential duration at 50% repolarization; APD90, action potential duration at 90% repolarization; BCL, basic cycle length; DADs, delayed afterdepolarizations; EADs, early afterdepolarizations; LV, left ventricular PV, pulmonary veins; TA, triggered activity; TOP, take-off potential

Section: Cellular and Molecular
ABSTRACT

Objectives: To examine the electrophysiologic and antiarrhythmic effects of the new antiarrhythmic agent AZD1305 in canine pulmonary vein (PV) sleeve preparations isolated from untreated and chronic amiodarone-treated animals. Background: Ectopic activity arising from PV sleeves plays a prominent role in the development of atrial fibrillation (AF). Delayed afterdepolarizations (DADs) and late phase 3 early afterdepolarizations (EADs), originating from PV have been proposed as potential triggers in initiation of AF. Methods: Action potentials were recorded from canine superfused PV sleeves using standard microelectrode techniques. Acetylcholine (ACh, 1 µM), isoproterenol (1 µM), or their combination was used to induce EADs, DADs and triggered activity (TA). The effects of AZD1305 (0.1-10 µM) were evaluated in PV sleeve preparations isolated from untreated and amiodarone-treated (40 mg/kg daily for 6 weeks) dogs. Results: AZD1305 (0.1-10 µM, 30 min) significantly prolonged action potential duration and reduced excitability. Abbreviating basic cycle length from 1000 to 300 ms resulted in a decrease of $V_{max}$ from $314\pm79$ to $251\pm55$ V/s ($\Delta=-20\%$) in control and from $177\pm53$ to $76.5\pm33$ V/s ($\Delta=-57\%$) following AZD1305 (n=6, p<0.05). AZD1305 markedly attenuated or suppressed DADs and DAD-induced TA, but not late phase 3 EADs. AZD1305-induced attenuation of excitability, leading to activation failure at much longer cycle lengths, was much more pronounced in PV from amiodarone-treated dogs. Conclusions: Potent effects of AZD1305 to depress excitability, prolong action potential duration and suppress DAD-induced triggered activity in canine PV sleeve preparations may be effective in suppressing triggers responsible for the genesis of AF and other atrial arrhythmias.
INTRODUCTION

Clinical and experimental studies have shown that arrhythmogenic activities originating from pulmonary veins (PV) (Haissaguerre, et al., 1998) are potential triggers in the initiation of AF (Burashnikov and Antzelevitch, 2006; Patterson, et al., 2005; Patterson, et al., 2006; Wongcharoen, et al., 2007; Lo, et al., 2007).

AZD1305 ((tert-butyl)2-[7-[2-(4-cyano-2-fluorophenoxy)ethyl]-9-oxa-3,7-diazabicyclo[3.3.1]non-3-yl]ethyl)carbamate, Figure 1) is a combined ion channel blocker that has undergone clinical testing for restoration and maintenance of sinus rhythm in AF patients. In vitro studies in transfected mammalian cells and ventricular cardiomyocytes show that AZD1305 predominantly blocks the rapid component of the delayed rectifying potassium current (I_{Kr}), the L-type calcium current and the inward sodium current, actions that all contribute to its functional effects on action potential duration, refractoriness and conduction (Andersson, et al., 2010; Carlsson, et al., 2009). Interestingly, this ion channel-blocking profile seems to translate into atrial predominant electrophysiological actions as well as a low potential for ventricular proarrhythmia such as torsade de pointes (Andersson, et al., 2010; Burashnikov, et al., 2009; Carlsson, et al., 2009). Amiodarone is commonly used for the treatment of AF and is likely the most effective pharmacological option for maintenance of sinus rhythm. The objective of the present study was twofold. Firstly, the study was designed to evaluate the electrophysiologic and antiarrhythmic effects of AZD1305 in canine superfused PV sleeve preparations isolated from untreated dogs. Secondly, preparations isolated from dogs chronically treated with amiodarone were studied to assess potential additive or synergistic electrophysiological actions of the two compounds. Preliminary data have been presented in abstract form (Sicouri, et al., 2008b).
METHODS

This investigation conforms to the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No 85-23, Revised 1996) and was approved by the Animal Care and Use Committee of the Masonic Medical Research Laboratory.

Six adult mongrel dogs were orally treated with amiodarone (40 mg/kg/day) for 6 weeks. The dose of amiodarone used is considerably higher than the regimen typically used clinically for management of AF but falls within the range of doses previously used in dog studies (Abdollah, et al., 1990; Gallagher, et al., 1989; van Opstal, et al., 2001). Serum concentrations of amiodarone measured using these high dosage regimens (Sicouri, et al., 1997) fell within the clinically effective concentration range (Holt, et al., 1986), supporting the appropriateness of the high oral dosing. No signs of hypothyroidism (marked bradycardia, lethargic state, lack of appetite), hyperthyroidism (tachycardia, excitation) or lung toxicity (breathing disability) were observed over the course of the study.

In the present study untreated or chronic amiodarone-treated for 6 weeks) dogs were anticoagulated with heparin (180 IU/kg) and anesthetized with sodium pentobarbital (35 mg/kg, IV). The chest was opened via a left thoracotomy and the heart excised and placed in a cold cardioplegic solution ([K⁺]₀ = 8 mmol/L, 4°C).

**Superfused pulmonary vein sleeve preparation**

PV sleeve preparations (approximately 2.0 x 1.5 cm) were isolated from left canine atria. The thickness of the preparation was approximately 2 mm. Left superior PVs were used preferentially in most experiments. The preparations were placed in a small tissue bath and superfused with Tyrode's solution of the following composition (mM): 129 NaCl, 4 KCl, 0.9 NaH₂PO₄, 20 NaHCO₃, 1.8 CaCl₂, 0.5 MgSO₄, 5.5 glucose, buffered with 95% O₂/5% CO₂ (35 ±
0.5°C). The PV preparations were stimulated at a basic cycle length (BCL) of 1000 ms during the equilibration period (1h) using electrical stimulation (1-3 ms duration, 2.5 times diastolic threshold intensity) delivered through silver bipolar electrodes insulated except at the tips. Transmembrane potentials were recorded (at a sampling rate of 41 kHz) using glass microelectrodes filled with 3 M KCl (10-20 MΩ DC resistance) connected to a high input-impedance amplification system (World Precision Instruments, model KS-700, New Haven, CT). The following parameters were measured: take-off potential (TOP), action potential amplitude (APA), action potential duration at 50 and 90% repolarization (APD₅₀ and APD₉₀), maximal rate of rise of action potential upstroke (V_max), and the minimum cycle length required to maintain 1:1 activation. The TOP was used instead of the resting membrane potential because of the slow phase 3 of the action potential of the PV sleeve preparation which does not return to the resting potential at the shortest BCLs. Acetylcholine (ACh, 1 μM), isoproterenol (1 μM), or their combination were used to induce late phase 3 EADs, DADs and triggered activity. The combination of parasympathetic and sympathetic stimulation has been shown to facilitate the development of late phase 3 EADs in PV sleeve preparations (Burashnikov and Antzelevitch, 2006; Patterson, et al., 2005; Patterson, et al., 2006), whereas sympathetic stimulation is known to lead to calcium overload, a condition responsible for the development of DADs (Chen, et al., 2000; Chen and Chen, 2006). DADs or EADs were elicited using stimulation trains of 20 beats introduced at progressively faster rates followed by a pause.

**Drugs:** Amiodarone (Cordarone®, 200 mg TAB) was obtained from Wyeth Pharmaceuticals, Vonore, TN, USA. AZD1305 (AstraZeneca R&D Mölndal, Sweden) was used at concentrations of 0.1 to 10.0 μM, a concentration range covering anticipated effective plasma concentrations for restoration and maintenance of sinus rhythm in patients (0.5 to 3 μM).
Statistics: Results are presented as mean ± SD. Statistical analysis was performed using one way repeated measures analysis of variance (ANOVA) followed by Bonferroni’s test. Mean values were considered to be different at p < 0.05.

RESULTS

Effects of AZD1305 on action potential characteristics of pulmonary vein sleeve preparations

Figure 2 shows a representative example of the effects of AZD1305 (10 μM) on action potential morphology and V_max of a PV sleeve preparation. AZD1305 induced a marked use-dependent decrease in V_max and 2:1 activation failure at a BCL of 200 ms. Composite data (n=6) of the effects of AZD1305 on V_max are shown in Figure 3 and Table 1. AZD1305 (3 and 10 μM) induced a significant decrease in V_max, APA and TOP and a significant increase in APD_{90}. The effects of AZD1305 were more pronounced at the faster rates of stimulation (use-dependent effect) and at higher concentration of AZD1305.

Effects of AZD1305 on excitability

Figure 4 illustrates composite data of the effects of AZD 1305 on the BCL at which 1:1 activation was lost (n=5). AZD1305 (3 and 10 μM) induced a significant increase of the BCL required to maintain 1:1 capture, indicating that the drug markedly depressed excitability.

Effect of AZD1305 on delayed and early afterdepolarizations (DADs and EADs)

In another series of experiments we investigated the effects of AZD1305 on EADs, DADs and TA provoked by isoproterenol in PV sleeve preparations. A representative experiment of the effects on DAD-induced triggered activity is shown in Figure 5. Isoproterenol-induced DAD activity was apparent following a train of 20 beats at BCLs of 300 and 150 ms. The DAD...
reached threshold at a BCL of 120 ms, giving rise to a triggered response followed by a prominent DAD. AZD1305 (3 μM) suppressed the triggered response and reduced the amplitude of the DADs. DAD-induced triggered activity was suppressed by 3 μM AZD1305 in 5 of 5 preparations. Late-phase 3 EADs induced by ACh or a combination of ACh and isoproterenol were largely unaffected by AZD1305 (n=3).

Effect of AZD1305 on chronic amiodarone-treated PV sleeve preparations

Figure 6 illustrates composite data of the effects of AZD1305 on the BCL at which 1:1 activation failed in PV sleeves isolated from untreated and amiodarone-treated dogs. In untreated animals AZD1305 (3 and 10 μM) led to a significant increase of the BCL required to maintain 1:1 activation, from 124 ms under control conditions to 165 and 246 ms following 3 and 10 μM AZD1305, respectively. In preparations from chronic amiodarone-treated dogs, AZD1305 (1 μM) dramatically increased the BCL required to maintain 1:1 activation, from 420 ms with chronic amiodarone alone to 1060 ms following the addition of 1 μM AZD1305. Thus, in PV sleeve preparations, pre-treatment with chronic amiodarone potentiates the effect of AZD1305 to depress excitability, suggesting a synergistic effect of AZD1305 and chronic amiodarone. In chronic amiodarone-treated PV preparations, late phase 3 EADs and DAD-induced triggered activity were rarely observed in the presence of isoproterenol and/or ACh (even at higher concentrations). Hence, the effect of AZD1305 on EADs and DADs could not be adequately tested due to the protective effect of chronic amiodarone.
DISCUSSION

Our results indicate that in PV sleeve preparations, AZD1305 exerts potent concentration-dependent effects to depress excitability, prolong action potential duration and suppress DAD-induced triggered activity. We observed a significant use-dependent decrease in $V_{\text{max}}$, action potential amplitude and take-off potential and a significant increase in action potential duration. These actions of AZD1305 are presumably secondary to its frequency-dependent blockade of the fast sodium current and the $I_{Kr}$ current as demonstrated in mammalian cell lines expressing the human isoforms of the ion channel proteins (Carlsson, et al., 2009). In addition, the data show that in the PV sleeves isolated from the chronic amiodarone-treated dogs, AZD1305 acted synergistically to further depress excitability.

The effects of AZD1305 are similar to those described for its analogue AZD7009 which showed a high efficacy in restoring sinus rhythm in patients with long-lasting AF (Carlsson, et al., 2006; Geller, et al., 2009). In addition, AZD1305 has been shown to attenuate $I_{Kr}$ blockade-induced APD prolongation in rabbit isolated Purkinje fibers and to widen the QRS interval and suppress dofetilide-induced torsade de pointes in anesthetized rabbits (Carlsson and Andersson, 2009; Carlsson, et al., 2009), actions attributed to its high potency in blocking the L-type calcium current and the late (persistent) sodium current. Accordingly, in the canine left ventricular wedge preparation, AZD1305 significantly increased R wave width (a surrogate measure of conduction velocity and sodium current blockade) at the highest concentration tested (10 µM) and slightly delayed repolarization but spontaneous or programmed electrical stimulation-induced proarrhythmia was never observed (Di Diego, personal communication).

Effects of AZD1305 on DAD and EAD activity in PV sleeves
Previous studies have shown that DADs and late phase EADs-induced TA can be easily induced in canine PV sleeve preparations following the addition of isoproterenol, ACh, high calcium alone or in combination (Sicouri, et al., 2008a; Sicouri, et al., 2009; Patterson, et al., 2005; Patterson, et al., 2006), in canine and rabbit isolated single PV myocytes (Chen, et al., 2000; Chen and Chen, 2006), and in canine coronary-perfused right atrial preparations (Burashnikov and Antzelevitch, 2006; Burashnikov and Antzelevitch, 2003). Conditions permitting intracellular calcium loading facilitate the development of DADs and late phase 3 EADs (Burashnikov and Antzelevitch, 2006). The present study shows that AZD1305 is capable of suppressing DAD-induced triggered responses in PV sleeve preparations. However, late-phase 3 EADs were largely unaffected by the drug. The effects of AZD1305 on DAD-induced triggered activity are similar to those of ranolazine (Sicouri, et al., 2008a).

**Synergistic effects of amiodarone and AZD1305**

In PV sleeve preparations isolated from amiodarone-treated dogs, AZD1305 markedly depresses excitability (Figure 6). The shortest BCL at which 1:1 activation could be maintained was dramatically increased from 420 to 1460 ms following the addition of low dose of AZD1305 (1 μM), pointing to a synergistic effect of the drug combination. Similar synergistic use-dependent depression of excitability was recently reported for the combination of chronic amiodarone and acute ranolazine in the canine isolated PV preparation (Sicouri, et al., 2010). Furthermore, the drug combination effectively suppressed triggered activity in the PV sleeve preparations.

The synergism of chronic amiodarone and AZD1305 may be due to their interaction with different states of the cardiac sodium channel. Amiodarone is an inactivated-state blocker of cardiac sodium channels (Whalley, et al., 1995; Kodama, et al., 1999). If AZD1305 is a
predominantly activated state blocker of the sodium channel, the drug combination could lead to a synergistic depression of $I_{Na}$ by blocking the channel during both activated and inactivated states. In support of this hypothesis, our preliminary voltage clamp data indicate that AZD1305 interacts most strongly with the open or activated state of the sodium channel (Burashnikov et al. unpublished observation). A synergistic electrophysiological action between amiodarone and a combined ion channel blocker like AZD1305 may be effective in suppressing AF, as has been demonstrated for the combination of amiodarone and ranolazone (Antzelevitch C, Burashnikov A, Sicouri S, Carlsson L. International Patent Application No. PCT/SE2009/051312). However, before embarking on such studies it has to be demonstrated that any potential drug combination does not exacerbate the risk of repolarization delay and torsade de pointes. Whether amiodarone-treated patients can be safely converted with any repolarization-delaying agent is still unclear. A few clinical studies have demonstrated that restoration of sinus rhythm by ibutilide in patients receiving amiodarone is equally safe (and effective) as the use of ibutilide alone (Fragakis, et al., 2005; Glatter, et al., 2001). In the canine left ventricle, long-term treatment with amiodarone differentially altered the cellular electrophysiology of the ventricular myocardium such that the transmural dispersion of repolarization decreased (Sicouri, et al., 1997). Furthermore, amiodarone dramatically decreased the effect of d-sotalol to exaggerate dispersion of repolarization or to induce repolarization-related proarrhythmia.

In conclusion, the actions of AZD1305 to depress excitability, prolong action potential duration and eliminate TA in PV sleeve preparations may be effective in suppressing the triggers responsible for the development of AF and other atrial arrhythmias.
Acknowledgements

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FOOTNOTES:

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FIGURE LEGENDS

Figure 1. Molecular structure of AZD1305.

Figure 2. Effects of AZD1305 on action potential morphology and maximum rate of rise of action potential upstroke (V_max) in a pulmonary vein (PV) sleeve preparation. Upper panel: Control recordings. Lower panel: Effect of AZD1305 (10 μM). Basic cycle length (BCL): 1000, 300, and 200 ms. AZD1305 induces a marked use-dependent decrease in V_max and 2:1 activation failure at BCL 200 ms. V/s; volt per second.

Figure 3. Composite data of the effects of AZD1305 on action potential parameters of PV sleeve preparations n=6. A. Effects on maximal rate of rise of the action potential upstroke (V_max). B. Effects on action potential duration measured at 90% repolarization (APD_{90}). C. Effects on action potential amplitude (APA). D. Effects on take-off potential (TOP). AZD1305 (3 and 10 μM) causes a significant decrease in V_max, APA and TOP and significantly increases APD_{90}. * p<0.05 AZD1305 vs Control.

Figure 4. Effect of AZD1305 on the briefest basic cycle length (BCL) at which 1:1 activation is maintained in the PV sleeve preparations. (n=5). AZD1305 (3 and 10 μM) significantly increases the BCL at which 1:1 is maintained. * p<0.05 AZD1305 vs. Control.

Figure 5. Effect of AZD1305 on delayed afterdepolarizations (DADs) and triggered activity induced by isoproterenol in a PV sleeve preparation. Upper panel: effect of isoproterenol (1 μM). DAD activity is apparent following a train of 20 beats at basic cycle length (BCL) of 300 and 150 ms. The DAD reaches threshold at a BCL of 120 ms, giving rise to a triggered response.
followed by a prominent DAD. Lower panel: AZD1305 (3 μM) suppresses isoproterenol-induced triggered response and reduces the amplitude of the DADs.

**Figure 6.** Effect of AZD1305 on the briefest basic cycle length (BCL) at which 1:1 activation is maintained in PV sleeve preparations isolated from untreated and chronic amiodarone-treated dogs. AZD1305 (3 and 10 μM) causes a significant increase in the BCL required to maintain 1:1 activation. Pre-treatment with amiodarone significantly potentiates the effect of AZD1305. *p<0.05 vs Control, # p<0.05 amiodarone+AZD1305 vs amiodarone alone.
Table 1. Effects of AZD1305 on action potential duration at 50% and 90% repolarization (APD<sub>50</sub> and APD<sub>90</sub>), maximal rate of rise of the action potential upstroke (V<sub>max</sub>), action potential amplitude (APA) and take-off potential (TOP) in PV sleeves preparations at basic cycle lengths (BCLs) of 1000, 500, 300 and 200 ms.

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<th>V&lt;sub&gt;max&lt;/sub&gt; (V/sec)</th>
<th>APA (ms)</th>
<th>TOP (mV)</th>
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<td>113.7 ± 3.2</td>
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<td>105.8 ± 5.2*</td>
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<tr>
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<td>177.83 ± 53.4*</td>
<td>98.0 ± 5.4*</td>
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<td>10.0 μM</td>
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<td>81.7 ± 12.1</td>
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n=6, *p<0.05, AZD1305 vs Control.
AZD1305

Figure 1
Figure 2

A graph showing the effect of different BCL values (1000 ms, 300 ms, and 200 ms) on the voltage (V) response. The graphs demonstrate the maximum voltage change (V_max) for each BCL:

- BCL = 1000 ms: V_max = 249 V/s
- BCL = 300 ms: V_max = 245 V/s
- BCL = 200 ms: V_max = 221 V/s

The graph also illustrates the impact of AZD1305 (10 μM) on the voltage response, showing activation failure at a ratio of 2:1.
Figure 4

- **Control**
- AZD1305 1 μM
- AZD1305 3 μM
- AZD1305 10 μM

BCL (ms)

- Control: 124 ms
- AZD1305 1 μM: 148 ms
- AZD1305 3 μM: 165 ms
- AZD1305 10 μM: 246 ms

* indicates a significant difference compared to the control.
Figure 5

BCL = 300 ms

Isoproterenol (1 μM)

+ AZD1305 (3 μM)

150 ms

120 ms

200 ms

mV
Figure 6

- Control
- AZD1305 3 μM
- AZD1305 10 μM
- Amiodarone
- Amiodarone + AZD1305 1 μM

BCL (ms)

- 124
- 165
- 246
- 420
- 1060

* #