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HMR 1098 is not an SUR isotype specific inhibitor of heterologous or sarcolemmal K_{ATP} channels

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Abstract

Murine ventricular and atrial ATP-sensitive potassium (K_{ATP}) channels contain different sulfonylurea receptors (ventricular K_{ATP} channels are Kir6.2/SUR2A complexes, while atrial K_{ATP} channels are Kir6.2/SUR1 complexes). HMR 1098, the sodium salt of HMR 1883 {1-[[5-[2-(5-chloro-*o*-anisamido)ethyl]-2-methoxyphenyl]sulfonyl]-3-methylthiourea}, has been considered as a selective sarcolemmal (i.e. SUR2A-dependent) K_{ATP} channel inhibitor. However, it is not clear whether HMR 1098 would preferentially inhibit ventricular K_{ATP} channels over atrial K_{ATP} channels. To test this, we used whole-cell patch-clamp techniques on mouse atrial and ventricular myocytes as well as $^{86}\text{Rb}^+$ efflux assays and excised inside-out patch-clamp techniques on Kir6.2/SUR1 and Kir6.2/SUR2A channels heterologously expressed in COSm6 cells. In mouse atrial myocytes, both spontaneously activated and diazoxide-activated K_{ATP} currents were effectively inhibited by 10 μM HMR 1098. By contrast, in ventricular myocytes, pinacidil-activated K_{ATP} currents were inhibited by HMR 1098 at a high concentration (100 μM) but not at a low concentration (10 μM). Consistent with this finding, HMR 1098 inhibits $^{86}\text{Rb}^+$ effluxes through Kir6.2/SUR1 more effectively than Kir6.2/SUR2A channels in COSm6 cells. In excised inside-out patches, HMR 1098 inhibited Kir6.2/SUR1 channels more effectively, particularly in the presence of MgADP and MgATP (mimicking physiological stimulation). Finally, dose-dependent enhancement of insulin secretion from pancreatic islets and decrease of blood glucose level confirm that HMR 1098 is an inhibitor of Kir6.2/SUR1-composed K_{ATP} channels.

Keywords

nucleotide; sulfonylurea; cardiomyocyte; insulin; patch clamp

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INTRODUCTION

ATP sensitive potassium (K_{ATP}) channels are expressed in most excitable tissues, including heart, skeletal and smooth muscle, neurons and pancreatic β cells [1]. K_{ATP} channels are hetero-octamers of four pore-formed inwardly rectifying K^+ channels (Kir6.1 or Kir6.2), each Kir6.x subunit being associated with one ATP-binding cassette sulfonylurea receptor (SUR1 or SUR2) [2,3]. Different combinations of Kir6.x and SURx subunits generate tissue specific channels in different tissues. In pancreatic β cells, K_{ATP} channels are Kir6.2/SUR1 complexes [4]. In ventricular cardiomyocytes, K_{ATP} channels are primarily Kir6.2/SUR2A complexes [5], however, multiple studies have demonstrated the presence of SUR1 transcripts and protein, as well as pharmacological effects of diazoxide in mouse, rat, and human myocytes [6-9]. At least in the mouse, it is now clear that SUR1 is an essential component of atrial K_{ATP} channels [10,11]. Moreover it has been demonstrated that levels of different transcripts and pharmacological properties change with different disease states [9,12]. The differential subunit make-up in different tissues provides the appealing possibility of subunit-specific and hence tissue-specific pharmacological modulation of channel activity. It is well-established that diazoxide is an effective K_{ATP} channel opener in the pancreas, but is ineffective in ventricular sarcolemma, whereas pinacidil is an effective opener in the ventricle, but is ineffective in the β -cell, or in the mouse atrium [10,13-15].

Based on early studies, the sulfonylurea HMR 1883 {1-[[5-[2-(5-chloro-*o*-anisamido)ethyl]-2-Methoxy phenyl]sulfonyl]-3-methylthiourea} was reported to be a cardioselective K_{ATP} channel inhibitor both *in vitro* and *in vivo* [16,17]. Later, HMR 1098 (Figure 1), the sodium salt of HMR 1883, was subsequently reported to prevent rilmakalim-induced reduction in action potential duration in human cardiomyocytes [18], and moreover to selectively inhibit heterologously expressed Kir6.2/SUR2A channels versus Kir6.2/SUR1 channels [19,20]. Furthermore, HMR 1098 was suggested to be antiarrhythmic in rats and rabbits [21,22]. Based on these studies, this sulfonylurea has since been widely used as a specific SUR2A-based sarcolemmal K_{ATP} channel inhibitor, and used in whole heart studies to differentiate effects of SUR2A-based sarcolemmal channel block from block of mitochondrial or other channels [23-28]. The realization that in mouse heart, the atrial K_{ATP} is SUR1-based, raises the question whether HMR1098 will only act on SUR2A-dependent ventricular channels. To test this, we used whole-cell patch-clamp techniques on mouse atrial and ventricular myocytes, as well as excised inside-out patch-clamp techniques and $^{86}Rb^+$ efflux assays on Kir6.2/SUR1 and Kir6.2/SUR2A channels heterologously expressed in COSm6 cells. Our results indicate that HMR 1098 actually inhibits atrial K_{ATP} channels more effectively than ventricular K_{ATP} channels and this surprising finding is paralleled by more potent inhibition of heterologously expressed Kir6.2/SUR1 than Kir6.2/SUR2A channels, as well as effective stimulation of β -cell insulin secretion and decrease in blood glucose level *in vivo*. These results lead to the clear-cut conclusion that HMR 1098 is not SUR2A-, nor cardiac specific K_{ATP} channel inhibitor.

METHODS

All protocols were approved by the Animal Studies Committee at Washington University School of Medicine.

Cardiomyocyte isolation

Cardiomyocytes were isolated from 3-5 months old C57BL mice. Briefly, mice were anesthetized using 2.5 % Avertin (2-2-2 Tribromoethanol, 10 ml/kg mouse). The heart was excised with the ascending aorta and immersed in cold calcium-free Wittenberg Isolation Medium (WIM) containing (in mM): 116 NaCl, 5.4 KCl, 8 MgCl₂, 1 NaH₂PO₄, 1.5 KH₂PO₄, 4 NaHCO₃, 12 Glucose, 21 N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic

acid) (HEPES), 2 Glutamine plus essential vitamins (GIBCO) and essential amino acids (GIBCO) (pH 7.40). After brief rinse in cold WIM, the heart was cannulated through the aorta, attached to a Langendorff perfusion system and perfused with WIM for 5 min at 37°C, followed by 20 min perfusion of WIM containing 270 units/ml collagenase type 2 (Worthington Biochemical) and 10 μ M CaCl_2 at 37°C. The heart was then transferred to WIM containing 50 mg/ml BSA, 12.5 mg/ml taurine and 150 μ M CaCl_2 . The ventricles were chopped into small pieces and triturated with a fire-polished pipette to dissociate into a single ventricular myocyte suspension. Both atrial appendages were further incubated for 40 min at 37°C in WIM containing 270 units/ml collagenase and 0.8 units/ml elastase. After digestion, the atrial appendages were transferred to a KB solution containing (in mM): 20 KCl, 10 KH_2PO_4 , 20 Taurine, 10 K_2EGTA , 25 Glucose, 10 L-Glutamate, 40 Mannitol, 10 γ -amino-butyrate and 0.1% bovine serum albumin (pH 7.40), and triturated by a fire-polished pipette to dissociate into single atrial myocytes.

Expression of K_{ATP} channels in COSm6 Cells

COSm6 cells, cultured in COS media (High Glucose Dulbecco's Modified Eagle Medium (DMEM-HG), supplemented with 10% Fetal Calf Serum (FCS) and antibiotic (100 units/ml penicillin + 0.2 mM streptomycin)), were plated on cover slips in 6-well plates for excised inside-out patch experiments and on 12-well plates for $^{86}\text{Rb}^+$ efflux experiments. A mixture of Kir6.2 (in pcDNA3.1- vector), either SUR1 or SUR2A (both in pECE vector), and EGFP (in pEGFP-c1 vector) at a ratio of 3:5:2 respectively was incubated in 100 μ l DMEM and FUGENE 6 transfection reagent for 30 minutes and immediately applied to cells.

Electrophysiology

All electrophysiological studies were performed at room temperature.

Whole-cell patch clamp recording— K_{ATP} currents were recorded from cardiomyocytes using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA), a Digidata 1322A digitizer board (Molecular Devices, Sunnyvale, CA), and a MP-225 micromanipulator (Sutter Instrument Co., Novato, CA). Cardiomyocytes were continuously perfused with extracellular solution containing (in mM): 137 NaCl, 5.4 KCl, 0.5 MgCl_2 , 3 NaHCO_3 , 0.2 NaH_2PO_4 , 5 HEPES and 10 Glucose (pH 7.40), with additions as described. Patch clamp electrodes had 1-2 M Ω resistance when filled with pipette solution (in mM): 130 K-aspartate, 20 KCl, 4 K_2HPO_4 , 1 MgCl_2 , 10 ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 1 K_2ATP , and 10 HEPES (pH 7.2-7.30). K_2ATP was omitted from the pipette solution when spontaneously activated K_{ATP} currents were studied in atrial cardiomyocytes. Spontaneously activated K_{ATP} currents, and pinacidil- or diazoxide-activated K_{ATP} currents were assessed using a voltage ramp from -120 mV to +40 mV at a rate of 40 mV s^{-1} from a holding potential of -70 mV. Series resistance compensation was set at 70-90%, and the 4-pole low pass Bessel filter on the amplifier was set at 2 kHz. Whole-cell currents were digitized at 10 kHz using pCLAMP 9 (Molecular Devices, Sunnyvale, CA). Current traces were analyzed using pCLAMP 9 software (Molecular Devices, Sunnyvale, CA) and K_{ATP} currents were typically measured at +40 mV.

Excised inside-out patch clamp recording—COSm6 cells were transferred to coverslips one day post-transfection and the cells that fluoresced green under UV illumination were patch-clamped on the two following days. All recordings were made at +50 mV pipette voltage corresponding to -50 mV membrane holding potential using symmetrical solutions in the pipette and bath. To minimize channel rundown, we used a phosphate-buffered solution containing (in mM): 140 KCl, 1 EGTA, 1 $\text{K}_2\text{-EDTA}$, and 4 K_2HPO_4 (pH 7.40). Alternatively, experiments recapitulating nucleotide stimulation (100

μM ATP, 500 μM ADP, 0.5 mM free Mg^{2+}) were performed in standard K_{INT} buffer (150 mM KCl, 10 mM HEPES, and 1 mM EGTA at pH 7.4) to prevent the chelating of Mg^{2+} that would otherwise occur in the phosphate/EDTA buffer.

Excised inside-out patch clamp experiments were performed using a microfluidic capillary chip-based platform (Dynaflow, Collectricon Inc). DF-16 ProII chips were used, allowing synchronized control of switching between 16 experimental solutions. Laminar flow at each solution outlet of the microfluidic chip prevents mixing, and a computer-controlled stepper motor is used to move the chip relative to the patch pipette, allowing for relatively rapid solution exchange around the membrane patch. All test concentrations were examined on a given patch, and the data from each patch will generate one dose-response curve. To minimize the effect of current rundown or current refreshment from ATP re-introduction in certain experiments, we repeated experiments on the same patches and corrected data by current changes over time.

HMR 1098 inhibition of the K_{ATP} current was determined by obtaining complete dose-response curves from single patches. The HMR 1098 dose-response curve from each patch in nucleotide-free solution was best fit to a two exponential Hill equation [29]:

$$I_{\text{rel}} = f / \left(1 + ([\text{HMR}] / k_{\text{high,HMR}})^H \right) + (1-f) / \left(1 + ([\text{HMR}] / k_{\text{low,HMR}})^H \right)$$

where $k_{\text{high,HMR}}$ represents the high-affinity component and $k_{\text{low,HMR}}$ the low-affinity component, I_{rel} is the current normalized to that in the absence of HMR 1098, $[\text{HMR}]$ is the HMR 1098 concentration, $k_{\text{high,HMR}}$ and $k_{\text{low,HMR}}$ are the concentrations producing half-maximal inhibition, H is the Hill coefficient, and f is the fraction of high affinity inhibition. HMR 1098 dose-response curve from each patch in the presence of nucleotides was best fit to the single exponential equation:

$$I_{\text{rel}} = (1-C) / \left(1 + ([\text{HMR}] / k_{1/2,\text{HMR}})^H \right) + C$$

where I_{rel} is the current normalized to that in the absence of HMR 1098, $[\text{HMR}]$ is the HMR 1098 concentration, $k_{1/2,\text{HMR}}$ is the concentration producing half-maximal inhibition. HMR concentration, C , a dynamic range index, and H the Hill coefficient.

$^{86}\text{Rb}^+$ Efflux Assay

The COS media was aspirated from the cells 24 hours post-transfection and replaced with fresh COS media containing $^{86}\text{RbCl}$ (1 $\mu\text{Ci/ml}$). Cells were incubated for an additional day to maximize channel expression and intracellular $^{86}\text{Rb}^+$ accumulation. Experiments were performed with Ringer's solution containing (in mM): 118 NaCl, 2.5 CaCl_2 , 1.2 KH_2PO_4 , 4.7 KCl, 25 NaHCO_3 , 1.2 MgSO_4 , and 10 HEPES (pH 7.4). Immediately prior to adding corresponding concentrations of HMR 1098 (in μM : 1, 10, 30, 100, 300) to separate cell wells from the same cell preparation, the cells were preincubated for 30 minutes with Ringer's solution containing metabolic inhibitors (1 mM 2-deoxy-D-glucose plus 3 μM oligomycin) to activate channel currents and then washed twice with the same solution, to remove external $^{86}\text{Rb}^+$. At the indicated time points, the solution was aspirated from each well to measure the radioactive content and replaced with fresh solution. After the final time point, cells were lysed with 1% SDS and the remaining $^{86}\text{Rb}^+$ was counted. Radioactivity at each time point is presented as cumulative $^{86}\text{Rb}^+$ efflux relative to total counts. Experiments

were repeated six times. HMR 1098 inhibition of the $^{86}\text{Rb}^+$ effluxes at the 40 min time point was best fitted to the logistic equation:

$$R(HMR) = R_{\max} \times IC_{50}^H / (IC_{50}^H + [HMR]^H),$$

where $R(HMR)$ is the relative efflux at a given $[HMR]$, $R_{\max}$ is the radioactivity in the absence of HMR 1098, IC_{50} is the concentration producing half-maximal inhibition, and H is the Hill coefficient.

Pancreatic islet isolation and insulin secretion measurement

C57BL wild-type mouse pancreatic islets were isolated as described previously [30]. Briefly, mice were anesthetized with halothane© (0.2 ml) in an anesthetizing chamber and killed by cervical dislocation. The bile duct was cannulated and perfused with Hank's balanced salt solution containing 70 units/ml collagenase. The pancreas was removed and digested for 5 min at 37°C, then washed 3 times in cold Hank's solution. Islets were isolated under a dissecting microscope and pooled islets were maintained in CMRL-1066 culture medium (GIBCO) supplemented with 10% FCS, 100 units/ml penicillin, and 0.2 mM streptomycin. Following overnight incubation in low glucose (5.6 mM) CMRL-1066 medium, islets (10 per well in 12 well plates) were pre-incubated in glucose-free CMRL-1066 medium plus 3 mM glucose, then incubated for 60 min at 37°C in low glucose CMRL-1066 medium without or with 1, 10, 30, 100 and 300 μM of HMR 1098. After the incubation period, the medium was removed and assayed for insulin secretion. Insulin secretion was measured by rat insulin radioimmunoassay (RIA) according to manufacturer's procedure (Millipore, St. Charles, MO). Experiments were repeated in triplicate from 4 mice. The insulin contents were normalized to those with 300 μM HMR 1098 and the dose-response curve of HMR 1098 was best fitted to the logistic equation:

$$I(HMR) = I_{\max} \times [HMR]^H / (EC_{50}^H + [HMR]^H),$$

Where $I(HMR)$ is the insulin contents to a given $[HMR]$, $I_{\max}$ is the insulin contents to a saturating $[HMR]$, EC_{50} is the concentration producing half-maximal effect, and H is the Hill coefficient.

“In vivo” blood glucose measurement

As described [31], fed C57BL wild-type mice were intraperitoneally injected with saline without or with 1, 10, 30, 300, and 3000 μg HMR 1098. Each group had 8 mice. Following injection, tail blood was taken at 0, 15, 30, 45, 60, 90, and 120 min and assayed for glucose content using the Glucometer Elite XL (Bayer).

Statistics

All data **are** presented as the mean $\pm$ standard error of mean (S.E.M.) with n being the number of cells/hearts (or patches in excised patch-clamp experiments), the number of independent experiments in $^{86}\text{Rb}^+$ efflux assay or in insulin secretion measurement, or the number of mice in blood glucose measurements. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* comparison of means and a two-tailed student t-test. Statistic significance was set as $P < 0.05$.

Materials

HMR 1098 was a gift from Aventis Pharma Deutschland GmbH. All other chemicals were obtained from Sigma (St. Louis, MO) unless indicated otherwise.

RESULTS

HMR 1098 inhibits atrial K_{ATP} channels more effectively than ventricular K_{ATP} channels

We investigated the effects of HMR 1098 on atrial K_{ATP} currents activated spontaneously or by K_{ATP} channel opener. Since murine sarcolemmal K_{ATP} channels are composed primarily of Kir6.2/SUR2A in mouse ventricle and Kir6.2/SUR1 in atrium [10,11], different sensitivities to HMR 1098 may be expected, given reported SUR2A selectivity [19,20]. First, we examined the effects on spontaneously activated atrial K_{ATP} currents as atrial K_{ATP} channels will automatically open in low intracellular ATP [32]. When an ATP-free pipette solution was used, spontaneously activated K_{ATP} currents reached peak in ~5 min, at which time, solution was switched to one containing 10 μ M HMR 1098. Ten μ M HMR 1098 significantly inhibited spontaneously activated atrial currents by ~85% ($n = 7$ cells from 3 hearts, Figure 2A & C). For these spontaneously activated atrial myocyte currents, it was not possible to examine the response to washout of HMR 1098 because the myocytes typically went into rigor during washout. Secondly, we activated atrial K_{ATP} currents by bath applying 100 μ M diazoxide, a SUR1-specific K_{ATP} channel opener, in cells voltage-clamped with 1 mM ATP in the pipette (see Methods). Following bath application of 100 μ M diazoxide, atrial K_{ATP} currents reached peak in ~8 min. Again, 10 μ M HMR1098 significantly inhibited these diazoxide-activated currents, by ~75% ($n = 6$ cells from 2 hearts), and in this case, significant current recovery was observed following drug removal (Figure 2B & C). These data indicate that 10 μ M HMR 1098 can potentially inhibit atrial K_{ATP} currents whether activated spontaneously or by K_{ATP} channel opener.

Next, we examined the effects of HMR 1098 on ventricular K_{ATP} channels. To activate K_{ATP} channels in ventricular myocytes, we bath applied a SUR2A-specific K_{ATP} channel opener-pinacidil. Peak current reached in ~15 minutes, at which time, solution was switched to one containing 100 μ M pinacidil plus HMR 1098 or glibenclamide for a subsequent 10 min. Although 10 μ M glibenclamide inhibited ventricular K_{ATP} current dramatically, at this concentration, HMR1098 was without effect ($n = 6$ cells from 2 hearts, Figure 3A & C), although currents were inhibited by ~65% at 100 μ M HMR1098 ($n = 6$ cells from 2 hearts), with partial recovery (to $68 \pm 5\%$ of control, $n = 4$ cells from 2 hearts) upon washout (Figure 3B & C). Therefore, HMR 1098 reversibly decreases ventricular K_{ATP} currents at 100 μ M, but not at 10 μ M, in contrast to the effectiveness of 10 μ M HMR 1098 on atrial myocytes.

HMR 1098 inhibits Kir6.2/SUR1 more effectively than Kir6.2/SUR2A channels in heterologously expressing system

Given reported SUR2A selectivity of HMR1098 [19,20], the above results are surprising, in suggesting that the drug is, if anything, acting preferentially on the SUR1-based atrial channels. To further examine SUR-selectivity, we co-transfected COSm6 cells with Kir6.2 and either SUR1 or SUR2A plasmids, and examined the effects of HMR 1098 on these heterologously expressed Kir6.2/SUR1 and Kir6.2/SUR2A channels. First, we examined the effects of HMR 1098 on $^{86}\text{Rb}^+$ effluxes through these channels. After loading with $^{86}\text{Rb}^+$, transfected COSm6 cells were incubated with metabolic inhibitors (MI) and HMR 1098 at different concentrations. HMR1098 dose-dependently inhibited MI-induced $^{86}\text{Rb}^+$ effluxes through Kir6.2/SUR1 channels and Kir6.2/SUR2A channels (Figure 4A & B). At the 40 min point, the apparent dose-response curves (Figure 4C) indicate more effective block of $^{86}\text{Rb}^+$ effluxes through SUR1-based ($IC_{50} = 33 \pm 3 \mu\text{M}$, $n = 6$) than SUR2A-based ($IC_{50} = 100 \pm 11 \mu\text{M}$, $n = 6$) channels, although direct comparison is complicated by the slower rate of

activation of SUR2A-based fluxes, which may reflect the lower sensitivity of SUR2A-based channels to metabolic inhibition [33].

We further performed excised inside-out patch clamp experiments to examine the effects of HMR 1098 on the currents through these heterologously expressed Kir6.2/SUR1 and Kir6.2/SUR2A channels. In Mg^{2+} -free conditions, 5 mM ATP completely inhibited K_{ATP} currents in excised inside-out patches from COSm6 cells transfected with Kir6.2/SUR1 channels or Kir6.2/SUR2A channels (Figure 5A & B). In this Mg^{2+} -free condition, HMR1098 inhibited both Kir6.2/SUR1 and Kir6.2/SUR2A currents with a biphasic inhibition curve, reflecting two drug binding sites [34]. The dose-response curves for HMR1098 inhibition were best fit by a two exponential equation ($2.3 \pm 0.7 \mu M$ (k_{high}), $2300 \pm 470 \mu M$ (k_{low}) for inhibition of Kir6.2/SUR1; $15 \pm 5 \mu M$ (k_{high}), $3000 \pm 680 \mu M$ (k_{low}) for inhibition of Kir6.2/SUR2A; $n = 9$), with the high affinity component accounting for ~50% in each case. It is apparent that high affinity inhibition by HMR1098 is slightly more effective on Kir6.2/SUR1 than Kir6.2/SUR2A channels ($P < 0.05$, Figure 5B).

In the presence of Mg^{2+} , as in physiological conditions inside cells, ATP and ADP have complex effects on K_{ATP} channel activity, both inhibiting the channel at the Kir6.2 subunit, and activating the channel through interactions with the SUR nucleotide binding folds [35]. Under these conditions, HMR 1098 block of both SUR1 and SUR2A-based currents was dominated by a high affinity component (Figure 5C), and again, HMR 1098 was slightly more effective on SUR1-based than SUR2A-based channels ($k = 2.1 \pm 0.6 \mu M$ for Kir6.2/SUR1 and $5.4 \pm 0.7 \mu M$ for Kir6.2/SUR2A, $P < 0.05$, $n = 9$), consistent with the higher sensitivity of atrial K_{ATP} channels to HMR 1098 than that of ventricular K_{ATP} channels. The Hill coefficients were 1.9 ± 1.0 and 1.7 ± 0.4 , respectively, for Kir6.2/SUR1 and Kir6.2/SUR2A channels.

HMR 1098 increases insulin secretion from pancreatic islets and decreases blood glucose level *in vivo*

Our results from cardiomyocytes and heterologously expressed channels suggest that HMR 1098 inhibits SUR1-based K_{ATP} channels more potently than SUR2A-based channels. Pancreatic β -cell K_{ATP} channels are some of the best characterized channels comprised of Kir6.2/SUR1 subunits. These channels regulate insulin secretion and thereby control blood glucose level. We tested the effects of HMR 1098 on insulin secretion from isolated pancreatic islets and blood glucose level *in vivo*. As predicted for inhibition of SUR1-based K_{ATP} channels, HMR 1098 potently increased insulin secretion from isolated pancreatic islets with an EC_{50} of $6.3 \pm 1.5 \mu M$ ($n = 3$) and a Hill coefficient of 1.1 ± 0.2 ($n = 3$) (Figure 6), confirming the non-specificity of the drug. In addition, 3000 μg HMR 1098 decreased blood glucose level from 152 to 110 mg/ml ($n = 8$, $p < 0.05$) in mice. Thus not only does HMR 1098 inhibit pancreatic β -cell K_{ATP} channels in isolated islets, but it can trigger insulin secretion *in vivo*.

DISCUSSION

The initially surprising finding of this study is that HMR 1098 is a more effective inhibitor of mouse atrial K_{ATP} channels than mouse ventricular K_{ATP} channels, which therefore reflects an effective inhibition of SUR1 isotype K_{ATP} channels [10,11] by HMR1098. This preference of HMR 1098 is confirmed directly by the results from heterologously expressed channels: HMR 1098 inhibits $^{86}Rb^{+}$ effluxes through Kir6.2/SUR1 channels more effectively than Kir6.2/SUR2A channels; HMR 1098 inhibits Kir6.2/SUR1 currents more effectively than Kir6.2/SUR2A currents in the presence of MgATP and MgADP. Furthermore, the dose-dependent enhancement of HMR 1098 of pancreatic islet insulin secretion and decrement of blood glucose level *in vivo* indicate HMR 1098 inhibition of

pancreatic K_{ATP} channels (ie, Kir6.2/SUR1). Therefore, HMR 1098 is not a SUR2A-specific, nor cardiac-specific, K_{ATP} channel inhibitor, under a variety of physiological conditions. The efficiency of HMR 1098 as a cardiac K_{ATP} channel inhibitor in experimental studies and clinical trials needs to be carefully evaluated in this light.

Reconciling conflicting interpretations of HMR1098 action: modulation by MgATP and MgADP

A line of studies has shown that HMR 1883 and HMR 1098 prevent action potential duration shortening induced by either K_{ATP} channel opener or ischemia in guinea pigs and pigs, and prevent ventricular fibrillation due to K_{ATP} activation in dogs, but reportedly without significant effects on plasma coronary blood flow, blood glucose or plasma insulin levels [16,17,36]. Furthermore, Manning-Fox *et al.* [19] reported that HMR 1098 was orders of magnitude more effective on SUR2A-composed ventricular K_{ATP} channels than SUR1-based INS-1 β cell K_{ATP} channels. These results were matched by experiments on recombinant Kir6.2/SUR2A and Kir6.2/SUR1 channels. Hence the conclusion of these studies was that HMR1098 is highly cardioselective and, indeed, SUR2A-selective, in complete contrast to the present conclusion, as well as that of Liu *et al.* [37] who showed that HMR1098 was an effective inhibitor of both Kir6.2/SUR1 (IC_{50} of 5 μ M) and Kir6.2/SUR2A (IC_{50} of 1.5 μ M) channels. It is therefore important to consider how to reconcile these disparate conclusions. Different potencies of HMR 1098 in different studies could arise from different recording conditions such as differences in species, recording protocols, temperatures, or metabolic states. The latter seems likely: the effects of intracellular nucleotides on K_{ATP} channel activity, and on sulfonylurea sensitivity, are quite complex.

In contrast to the much stronger effects of HMR on SUR1-based channels than SUR2A-based channels, indicated by the myocyte recordings and $^{86}Rb^+$ efflux experiments on transfected cells (Figs. 2-4), there is only a small difference between SUR1-based and SUR2A-based channels in excised patches (Fig. 5), although SUR1-based channels are still more sensitive in the presence or absence of nucleotides. The net effects of intracellular nucleotides on K_{ATP} channels are determined by the balance between their inhibition by binding to the Kir6.2 subunits and their stimulation (in forms of Mg-nucleotides-MgATP, MgADP) by binding to the nucleotide-binding domains (NBDs) of the SUR subunits. Moreover, it can be shown that in excised patches, sulfonylurea inhibition is biphasic, with a high affinity component, and a low affinity component, ascribed to actions on SUR or Kir6.2 respectively [34]. Previous studies have shown that Mg-nucleotides can enhance the inhibition of K_{ATP} channels by tolbutamide, meglitinide, and glibenclamide [38-42], at least in part by enhancing the high affinity component. The enhancement is suggested to be due to conformational changes caused by sulfonylurea binding to the SUR subunits that impair intracellular nucleotide-stimulation via the SUR subunits, and unmasking nucleotide inhibition on the Kir6.2 subunits [34,38,42].

It is thus important to make comparisons of drug sensitivity under comparable nucleotide conditions. In the Manning-Fox *et al.* study, high HMR1098 sensitivity was observed in ventricular membrane patches with 10 μ M MgATP present (their Figure 1A), and very low sensitivity in INS-1 cell membranes was obtained in the complete absence of ATP (their Figure 1B). In contrast, we examined the K_{ATP} opener-activated currents in myocytes with 1 mM ATP. Moreover, metabolic states of cardiomyocytes are variable even when obtained by the same isolation protocol. For example, the maximal response of ventricular K_{ATP} to pinacidil occurs anywhere between 5 minutes and half an hour, reflecting variable metabolic states of the cells; different metabolic states may lead to different results in otherwise comparable studies. Manning-Fox *et al.* also show that HMR 1098 has similar effectiveness in 0 ATP and 100 μ M ATP/100 μ M (their table 1). However, the dose-response curve was only examined over a narrow concentration range (0.1-100 μ M). By comparing HMR action

over a much wider range (1-3000 μ M) between 0 ATP and 100 μ M ATP/ 500 μ M ADP, our data reveal how the low affinity component becomes more prominent in the complete absence of nucleotides (Fig. 5), consistent with the findings of tolbutamide, meglitinide, and glibenclamide on K_{ATP} channels, [38-42]. Importantly, although it did not change blood glucose level in dogs at 3 mg/kg, iv [16, 17], our *in vivo* experiments show that at 3000 μ g (100 mg/kg, ip), HMR 1098 significantly decreases mouse blood glucose level *in vivo*. HMR 1098 inhibition of Kir6.2/SUR2A channels is reportedly reduced when activated by metabolic inhibition or low intracellular ATP [43], indicative of nucleotide-modulation of this drug sensitivity. Modulation of the efficiency of HMR 1098 by MgADP is quite apparent in our data from heterologously expressed channels. First, HMR 1098 potently inhibited $^{86}Rb^{+}$ efflux through both Kir6.2/SUR1 and Kir6.2/SUR2A channels when they were activated by metabolic inhibition, under which conditions, MgADP is expected to be elevated. Second, MgADP enhanced HMR 1098 inhibition of excised patch currents through both Kir6.2/SUR1 and Kir6.2/SUR2A channels, more prominently so for Kir6.2/SUR1 channels. Similar enhancement by Mg-nucleotides was reported for tolbutamide and glibenclamide inhibition of SUR1-composed K_{ATP} channels [34, 38, 41], and it was suggested that tolbutamide has a high-affinity site on SUR1 (but not on SUR2A) and a low-affinity site on Kir6.2. However, in contrast to the notion that the SUR binding site is isoform specific, our results suggest that HMR 1098 interacts with both SUR1 and SUR2A. The dose-inhibition relationships for Kir6.2/SUR1 and Kir6.2/SUR2A currents were best fitted by a two exponential equation in the absence of MgADP and MgATP, and the high affinity component became more prominent in the presence of MgADP in both cases. High- and low-affinity sites on both Kir6.2/SUR1 and Kir6.2/SUR2A were assigned for meglitinide block, but only low affinity sites for tolbutamide or glibenclamide on Kir6.2/SUR2A [41]. The absence of a benzamido moiety in tolbutamide and the existence of a cyclohexanic side chain on the benzenesulfonylurea portion of glibenclamide may underlie differences in potency.

Reinterpretation of HMR 1098: not a SUR2A-specific K_{ATP} channel inhibitor

It is not surprising that HMR 1098 effectively inhibits SUR1-composed K_{ATP} channels given its structural similarity to glibenclamide (Figure 1). Glibenclamide inhibits heterologously expressed Kir6.2/SUR1 and Kir6.2/SUR2A to a similar extent in excised inside-out patches [41], but inhibits Kir6.2/SUR1 more effectively than Kir6.2/SUR2A in the presence of MgADP [41] or in intact cells [14]. Similarly, the efficiency of HMR 1098 inhibition of SUR1- or SUR2A-composed K_{ATP} channel depends on metabolic states: HMR1098 inhibits Kir6.2/SUR1 more effectively, particularly in intact cells, and in excised patches in the presence of MgATP and MgADP.

As an assumed cardioselective and SUR2A-selective K_{ATP} channel inhibitor, HMR 1098 has been widely used to evaluate the involvement of sarcolemmal K_{ATP} channels in cardioprotection. Failure of HMR 1098 to block protection in some studies has been interpreted as excluding a role for sarcolemmal K_{ATP} channels in cardioprotection [44-48]. Similarly, lack of antiarrhythmic action of HMR 1098 in ischemia-reperfusion rat heart models [49] has been taken to exclude sarcolemmal K_{ATP} channels in ischemic arrhythmias. However, given the marked state-dependence of drug efficacy that has been shown before and in the present data, such studies require careful evaluation of the drug efficacy under the specific experimental conditions. Conversely, since HMR 1098 inhibition of SUR1-containing K_{ATP} channels increases markedly in the presence of MgADP, HMR 1098 at relatively low concentrations may selectively inhibit SUR1-containing atrial K_{ATP} channels [10] rather than ventricular K_{ATP} channels under certain metabolic conditions, and this might actually be a potential strategy for prevention of atrial-originated arrhythmias without side effects on ventricular action potentials under certain conditions. Again, since HMR

1098 inhibits pancreatic K_{ATP} channels, side effects on pancreatic function have to be considered when using HMR 1098 as a cardiac K_{ATP} channel inhibitor in vivo.

Finally, it is worth noting that, like other sulfonylureas, HMR 1098 exhibits a low affinity component of K_{ATP} inhibition (Fig. 5B), which is likely by direct inhibition of Kir6.2 subunits [34]. Though the low affinity inhibition is masked by the enhanced high affinity inhibition due to binding SUR subunits in the presence of Mg nucleotides, sulfonylureas could inhibit K_{ATP} channels in other non-targeted organs than pancreas by directly binding Kir6.2 subunits. Therefore, the non-targeted side effects of sulfonylureas should be considered when high concentration of sulfonylureas are used.

Conclusions

In conclusion, our data indicate that HMR 1098 is not a SUR2A-specific, nor cardiac-specific K_{ATP} channel inhibitor. Instead, HMR 1098 inhibits Kir6.2/SUR1 channels more effectively than Kir6.2/SUR2A channels in the presence of MgADP. In the mouse, which exhibits a strict chamber-specificity of SUR-isoform [10], this results in HMR 1098 being a more potent inhibitor of atrial K_{ATP} channels than ventricular K_{ATP} channels. Experimental and clinical studies using HMR 1098 should be very carefully evaluated in light of non-specificity for SUR isoforms, as well as the changing drug efficacy with metabolic states.

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Figure 1. Chemical structures of glibenclamide, HMR 1833, and HMR 1098. HMR 1098 is the sodium salt of HMR 1883. Both contain the same benzamido moiety as glibenclamide has, but a modified benzensulfonylurea part.

Figure 2.

HMR 1098 reversibly decreases atrial K_{ATP} currents at a low concentration (10 μ M) in whole-cell patch clamp recordings. **A)** 10 μ M HMR 1098 decreased K_{ATP} currents from an atrial myocyte activated spontaneously in an ATP-free pipette solution. The raw traces at the indicated time points 1 and 2 are shown at the bottom. **B)** 10 μ M HMR 1098 decreased K_{ATP} currents activated by 100 μ M diazoxide from an atrial myocyte. The raw traces at the indicated time points 1 and 2 are shown at the bottom. **C)** Summarized data show 10 μ M HMR 1098 decreased atrial K_{ATP} currents activated spontaneously in an ATP-free pipette solution or by diazoxide, and the HMR 1098 inhibition of diazoxide-activated currents was partially washout. All currents were normalized to the currents in the absence of HMR 1098 (Ctrl, control group). * $P < 0.05$ versus Ctrl, and # $P < 0.05$ versus Washout group by a one-way ANOVA followed by Tukey's post hoc comparison of means.

Figure 3.

HMR 1098 reversibly decreases ventricular K_{ATP} currents at a high concentration (100 μ M), but not at a low concentration (10 μ M) in whole-cell patch clamp recordings. **A)** 10 μ M HMR 1098 did not change K_{ATP} currents evoked by 100 μ M pinacidil from a ventricular myocyte. The upper figure shows that K_{ATP} currents were not changed during a 5 minute application of 10 μ M HMR 1098, but decreased by 10 μ M glibenclamide on the same cell. The raw traces for the indicated time points 1 and 2 are shown at the bottom. **B)** 100 μ M HMR 1098 decreased K_{ATP} currents from a ventricular myocyte. The upper figure shows that K_{ATP} currents were decreased by 100 μ M HMR 1098, and the raw traces for the indicated time points 1 and 2 are shown at the bottom. **C)** Summarized data show ventricular K_{ATP} currents are decreased by HMR 1098 at 100 μ M, but not at 10 μ M. The inhibition of 100 μ M HMR 1098 was reversible. All currents were normalized to the currents in the absence of HMR 1098 and glibenclamide (Ctrl, control group). * P <0.05 versus Ctrl and 10 μ M HMR group, and # P <0.05 versus Wash (washout) group by a one-way ANOVA followed by Tukey's post hoc comparison of means.

Figure 4.

HMR 1098 inhibits $^{86}\text{Rb}^+$ effluxes through Kir6.2/SUR1 channels more effectively than through Kir6.2/SUR2A channels heterologously expressed in COSm6 cells. **A)** Time course of HMR 1098 inhibition of $^{86}\text{Rb}^+$ effluxes through Kir6.2/SUR1 channels. The cells were pre-treated with metabolic inhibitors for 30 min and then co-applied with various concentrations (in μM) of HMR 1098 as indicated in the figure. **B)** Time course of HMR 1098 inhibition of $^{86}\text{Rb}^+$ effluxes through Kir6.2/SUR2A channels. The treatment was the same as indicated in A. **C)** Dose-response curves of HMR 1098 inhibition of $^{86}\text{Rb}^+$ effluxes through Kir6.2/SUR1 and Kir6.2/SUR2A channels at 40 min time point, showing much stronger inhibition of HMR 1098 on Kir6.2/SUR1 channels.

Figure 5.

HMR 1098 inhibits Kir6.2/SUR1 currents more effectively than Kir6.2/SUR2A currents of COSm6 cells in excised inside-out patch clamp recordings. **A)** Raw traces show HMR 1098 dose-dependently inhibited Kir6.2/SUR1 and Kir6.2/SUR2A currents by removing ATP or applying MgADP and MgATP. **B)** Dose-response curves of HMR 1098 inhibition of Kir6.2/SUR1 and Kir6.2/SUR2A currents activated by removing ATP from bath solution. **C)** Dose-response curves of HMR 1098 inhibition of Kir6.2/SUR1 and Kir6.2/SUR2A currents activated by MgADP and MgATP.

Figure 6.

HMR 1098 dose-dependently increases insulin secretion from isolated pancreatic islets and decreases blood glucose level *in vivo*. A) Dose-response curve of HMR 1098 enhancement on insulin secretion from mouse pancreatic islets. HMR 1098 increased insulin secretion with an EC_{50} of $6.3 \pm 1.5 \mu\text{M}$ ($n = 3$). The insulin contents were normalized to those with 300 μM HMR 1098. B) Time courses of blood glucose levels in mice after injection of various concentrations (in μg) of HMR 1098 as indicated in the figure. HMR 1098 at 3000 μg significantly decreased blood glucose level *in vivo* ($n = 8$).