Amiodarone (AMIO) was originally introduced more than 40 years ago for the treatment of angina, but it is now used therapeutically as an effective class III antiarrhythmia agent. AMIO is associated with numerous potential side effects, including pulmonary toxicity, hepatotoxicity, and thyroid dysfunction. Additionally, there have been reports of adverse drug interactions when AMIO is coadministered with any of a wide variety of other therapeutic agents, including theophylline, flecainide, cyclosporin A, and dextromethorphan. However, the most common—and potentially the most dangerous—drug interaction involving AMIO is its potentiation of the anticoagulant effect of warfarin, which greatly increases the patient’s risk for hemorrhage.

AMIO is frequently used for heart-rhythm control in patients with atrial fibrillation, with whom stroke is a major concern. Because warfarin is the preferred treatment for stroke prevention, AMIO is often coadministered with warfarin. An interaction always occurs between these two drugs, and this typically necessitates a dose reduction in warfarin of 25–40%, depending on the AMIO maintenance dose.

Early studies by Trager, O’Reilly, and co-workers demonstrated that AMIO inhibits the clearance of both (S)- and (R)-warfarin and causes greater decreases in the formation clearances of (S)-warfarin metabolites than in that of (R)-warfarin metabolites. However, AMIO proved to be a relatively weak inhibitor of (S)-warfarin metabolism in human liver microsomes (HLMs), whereas AMIO’s N-dealkylated metabolite, monodesethylamiodarone (MDEA), is 40–90 times more potent an inhibitor of CYP2C9 than the parent drug. These observations are significant because CYP2C9 is the major cytochrome P450 that terminates the pharmacologic activity of warfarin. Moreover, after administration of AMIO, MDEA is present in human plasma at a concentration approximately equal to that of the parent drug (~1–5 μmol/l). Indeed, there are in vivo data suggesting that plasma MDEA concentration may be a better predictor of the change in international normalized ratio (INR) than plasma AMIO concentration in patients receiving both drugs. These observations prompt the hypothesis that a major mechanism of the warfarin–AMIO interaction is inhibition of CYP2C9-mediated (S)-warfarin metabolism by MDEA.

Although MDEA is the major metabolite of AMIO in humans, several other metabolites have recently been identified in plasma samples of subjects receiving AMIO. The most prominent of these metabolites are 3′-hydroxy-N-monodesethylamiodarone (3′-OHMDEA),
Amiodarone metabolites in human plasma.

Figure 1

N, N-didesethylamiodarone (DDEA), and deaminated amiodarone (DAA). Another potential product of AMIO metabolism is O-desalkylamiodarone (ODAA). It is possible that one or more of these minor AMIO metabolites could contribute to the observed AMIO–warfarin interaction; indeed, several close analogs of ODAA have been shown to be low nanomolar inhibitors of CYP2C9. Delineation of specific inhibitory AMIO metabolites may also assist in a better understanding of the observed interindividual variability in the magnitude of this drug–drug interaction (DDI).

The research was carried out in three steps. First, a retrospective study was performed in a cohort of patients, who had undergone stable warfarin therapy, to determine the mean value and range of warfarin dose changes required for maintaining an INR of 2.0–3.0 after AMIO was added or discontinued. Next, stabilized plasma concentrations, [I], of AMIO and five of its circulating metabolites were determined in several patients undergoing combination therapy with warfarin and AMIO. To measure low metabolite concentrations in vivo, we developed a new, highly sensitive stable-label isotope liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay that necessitated the synthesis of deuterium-labeled internal standards. Finally, in vitro inhibition experiments were carried out with AMIO and its metabolites to measure the inhibition constant ($K_I$) against CYP2C9-mediated (S)-warfarin 7-hydroxylation in HLMs. To best assess the likelihood of an in vivo drug interaction, we measured the free fraction of AMIO and its various metabolites in HLMs and in plasma and calculated the respective $[I]_u/K_{I,u}$ values for the parent drug and its circulating metabolites.

RESULTS

In vivo effect of AMIO on warfarin dose requirement

We analyzed cases of out-of-range INR attributable to warfarin–AMIO interaction and occurring upon either addition or elimination of AMIO therapy in patients undergoing stable warfarin treatment. Over the 6-year period of the retrospective clinical evaluation, 73 case studies were identified that met these requirements. The results of the analysis are described in Table 1. The mean change in warfarin dose required to maintain an INR of
2–3 was 25.6%. The requirements for change in warfarin dose were highly variable, ranging from 5.9 to 65%.

**Synthesis of AMIO metabolite standards**

To enable the most rigorous quantitative assessment of AMIO metabolite concentrations in vivo and in vitro, we adopted stable-isotope methodology for all the LC-MS/MS analyses. Procedures for the synthesis of unlabeled AMIO and many of its known human plasma metabolites are described in the literature,27–30 but the syntheses of deuterium-labeled standards for AMIO, MDEA, DDEA, ODAA, and DAA are reported here for the first time. The deuterium source for isotope-labeled AMIO,7 as well as its deaminated6 and O-desalkylated5 derivatives, was p-methoxy-(2,3,5,6-d4)-benzoic acid, 1 (Figure 2a).

A slightly different methodology was used for the syntheses of the isotopically labeled analogs of MDEA and DDEA, 9 and 11, both of which were derived from unlabeled ODAA using 1,2-dibromo-d4-ethane as the deuterium source (Figure 2b). These procedures provided the required stable-isotope internal standards with good yield and purity and with high deuterium content (>95%) reflecting the characteristics of the labeled starting materials.

**Measurement of concentrations of AMIO parent drug and metabolites in human plasma**

The deuterated standards of AMIO and its metabolites were used to develop a LC-MS/MS assay for the quantitation of these compounds in the plasma samples from patients undergoing concomitant warfarin and AMIO therapy in a clinical setting (Figure 3). Blood was drawn from three patients at various time points up to 14 weeks after initiation of AMIO treatment. The concentration levels of the parent drug and its metabolites in plasma were seen to plateau at ~8 weeks after addition of AMIO therapy to a stabilized warfarin regimen (Figure 4). Therefore, the average plasma drug and metabolite concentrations were calculated for each of the patients between weeks 8 and 14, and these values were then averaged for the three patients to determine the mean steady-state plasma concentrations of AMIO, MDEA, DDEA, 3′-OHMDEA, DAA, and ODAA (1.09 ± 0.09, 1.16 ± 0.08, 0.059 ± 0.003, 0.42 ± 0.34, 0.015 ± 0.003, and 0.11 ± 0.01 μmol/l, respectively, after converting the units from ng/ml, Table 2). The values for AMIO, MDEA, 3′-OHMDEA, DDEA,
and DAA are similar to those reported in an earlier study by Ha et al.24 Plasma ODAA concentrations are reported here for the first time.

**Inhibition of (S)-warfarin 7-hydroxylation in HLMs**

Kinetic studies were carried out in pooled HLMs to determine the enzymatic mechanism of inhibition and to measure the $K_I$ values for the inhibition of (S)-warfarin metabolism by AMIO and its metabolites. Both ODAA (Figure 5a) and DAA (Figure 5b) were found to be purely competitive inhibitors of (S)-warfarin 7-hydroxylation, with $K_I$ values of 0.032 ± 0.006 and 2.0 ± 0.3 μmol/l, respectively, whereas AMIO, MDEA, DDEA, and 3′-OHMDEA appeared to be mixed inhibitors of warfarin metabolism in HLMs (Figure 5c–f). The respective $K_I$ values for these four nitrogen-bearing compounds, determined from Dixon plots, were calculated to be 39 ± 14, 5.9 ± 0.9, 0.053 ± 0.008, and 5.2 ± 2.0 μmol/l, respectively. Because of extensive nonspecific protein binding, half maximal inhibitory concentration values for the test compounds varied significantly, and linearly, with the amount of microsomal protein used in the incubation procedures (data not shown). Therefore, the kinetic estimates presented here are $K_I$ (apparent) values and need to be corrected for the free fractions available in metabolic incubation containing 0.25 mg/ml microsomal protein.

**Determination of fraction unbound ($f_u$) of parent drug and metabolites**

Protein binding experiments demonstrated that AMIO and the majority of its metabolites are very highly bound in both HLMs and human plasma. In experiments with HLMs, carried out at 0.25 mg/ml microsomal protein, $f_u$ values of 1.4 ± 0.2, 1.2 ± 0.4, 1.1 ± 0.3, and 1.7 ± 0.1% were calculated for AMIO, MDEA, DDEA, and DAA, and ODAA and 3′-OHMDEA exhibited $f_u$ values of 5.0 ± 2.0 and 17 ± 2.8%, respectively. Most of these compounds are more tightly bound to HLM protein than to plasma protein (plasma $f_u$ values were calculated as 3.3 ± 0.8, 2.0 ± 0.4, 4.2 ± 0.4, and 4.9 ± 1.7% for AMIO, MDEA, DDEA, and DAA, respectively). ODAA and 3′-OHMDEA do not fit this trend; both of these compounds are much more tightly bound to plasma protein than to microsomal protein (plasma $f_u$ values of 0.13 ± 0.01 and 1.8 ± 0.3%, respectively) (Table 2).

Similar protein binding experiments for these compounds were carried out in the presence of either 4 μmol/l (S)-warfarin, for studies in HLMs, or 2 μg/ml rac-warfarin (approximate physiological concentration), for studies in human plasma. Warfarin was found to have no effect on metabolite protein binding in either HLMs or plasma; the values of the unbound fractions remained essentially unchanged from the values...
reported above, showing less than twofold variation in all cases (data not shown).

**Determination of \([I]/K_I\) and \([I]_u/K_{Iu}\) for parent drug and metabolites**

\([I]/K_I\) is defined here as the total mean plateau concentration of the drug (or metabolite) in plasma (i.e., at 8–14 weeks after initiation of therapy) for the three test subjects divided by the \(i\) (apparent) value measured for the inhibition of (S)-warfarin 7-hydroxylation in HLMs. Using this definition, the values of \([I]/K_I\) were calculated to be 0.028, 0.20, 1.1, 3.4, 0.0076, and 0.081 for AMIO, MDEA, DDEA, ODAA, DAA, and 3′-OHMDEA, respectively (Table 2). \([I]_u/K_{Iu}\) is defined as the ratio of unbound plasma drug concentration \((\text{amino acid or metabolite concentration})\) to the microsomal \(K_I\) corrected for unbound microsomal drug concentration \((K_{Iu} = K_I \times f_u(mics))\). These values were determined to be 0.066, 0.33, 4.3, 0.089, 0.022, and 0.0086 for AMIO, MDEA, DDEA, ODAA, DAA, and 3′-OHMDEA, respectively (Table 2).

**DISCUSSION**

The warfarin–AMIO interaction is known to be due to metabolic inhibition of the P450 enzymes responsible for the clearance of warfarin, mainly CYP2C9. In patient studies, our results were in conformity with (i) the wide interindividual variability in the magnitude of the warfarin–AMIO interaction reported initially by Kerin et al. and (ii) the plasma concentration ranges of AMIO and several metabolites reported earlier by Ha et al. In addition, in these studies, ODAA was quantified (60.5 ± 5.6 ng/ml, 0.11 ± 0.01 μmol/l) for the first time in human plasma (although Deng and co-workers very recently identified ODAA in human bile and also as a product of metabolic incubation of AMIO with HLMs). The results we report here relating to the in vitro inhibition and protein binding experiments with AMIO and its metabolites serve as a means of elucidating details of the molecular mechanism underlying this in vivo interaction.

AMIO is known to be highly protein-bound in human plasma, and the \(f_u\) measured in plasma in this study (3.3 ± 0.8%) is in excellent agreement with the value reported previously by Laloz et al. (3.7 ± 0.6%). Both values were determined using the ultracentrifugation method for measuring free drug fraction. The plasma free fractions of the metabolites of amiodarone—MDEA, 3′-OHMDEA, DDEA, ODAA, and DAA, which varied from ~0.1 to 5%—have not previously been reported.

In kinetic experiments carried out in HLMs at 0.25 mg/ml microsomal protein the \(K_I\) values for the inhibition of (S)-warfarin 7-hydroxylation by AMIO, MDEA, 3′-OHMDEA, ODAA, DAA, and DAA, and which varied from ~0.1 to 5%—have not previously been reported.

In kinetic experiments carried out in HLMs at 0.25 mg/ml microsomal protein the \(K_I\) values for the inhibition of (S)-warfarin 7-hydroxylation by AMIO, MDEA, 3′-OHMDEA, ODAA, DAA, and DAA, and which varied from ~0.1 to 5%—have not previously been reported.

**Table 2** Comparison of \([I]/K_I\) and \([I]_u/K_{Iu}\) values for the inhibition of (S)-warfarin 7-hydroxylation by AMIO and its circulating human metabolites

| Inhibitor | \([I] (\mu mol/l)\) | \(K_I (\mu mol/l)\) | \(f_u(\%)\) | \(f_u(mics)(\%)\) | \(\text{amino acid or metabolite concentration}\) | \([I]/K_I\) | \([I]_u/K_{Iu}\) |
|-----------|-----------------|-----------------|--------------|--------------|-----------------------------------|------------|--------------|
| AMIO      | 1.09 ± 0.09†    | 39 ± 14         | 3.3 ± 0.8    | 1.4 ± 0.2    | 0.028                             | 0.066      |              |
| MDEA      | 1.16 ± 0.08     | 5.9 ± 0.9       | 2.0 ± 0.4    | 1.2 ± 0.4    | 0.20                              | 0.33       |              |
| DDEA      | 0.059 ± 0.003   | 0.053 ± 0.008   | 4.2 ± 0.4    | 1.1 ± 0.3    | 1.1                                | 4.30       |              |
| ODAA      | 0.11 ± 0.01     | 0.032 ± 0.06    | 0.13 ± 0.01  | 5.0 ± 2.0    | 3.4                                | 0.089      |              |
| DAA       | 0.015 ± 0.003   | 2.0 ± 0.3       | 4.9 ± 1.7    | 1.7 ± 0.1    | 0.008                             | 0.022      |              |
| 3′-OHMDEA | 0.42 ± 0.34     | 5.2 ± 2.0       | 1.8 ± 0.3    | 17 ± 2.8     | 0.081                             | 0.009      |              |

†Error measurements represent standard deviations of the mean.
metabolism in HLMs, whereas the amine compounds (AMIO, MDEA, 3′-OHMDEA, and DDEA) were mixed inhibitors of (S)-warfarin 7-hydroxylation (Figure 5). Ohyama et al. previously reported AMIO to be a noncompetitive inhibitor and MDEA to be a mixed inhibitor of (S)-warfarin metabolism in microsomes derived from human B-lymphoblastoid cells coexpressing CYP2C9 and P450 reductase, with \( K_i \) values of 95 and 2.3 \( \mu \text{mol/l} \), respectively.20

At present, the US Food and Drug Administration guidance for the use of in vitro data to predict in vivo DDIs relies on the comparison of total inhibitor plasma concentration to in vitro inhibition constant (\([I]/K_i\)), generally measured in microsomes.33 Compounds that exhibit \([I]/K_i\) values <0.1 are thought to have only a remote likelihood of causing an in vivo interaction; for compounds with an \([I]/K_i\) ratio in the range 0.1–1.0, interactions are considered possible, and for those with an \([I]/K_i\) > 1.0, interactions are considered likely.33,34 Using this measure, our data suggest that both DDEA and ODAA are likely to cause a DDI with warfarin, and MDEA is a possible contributor. There are, however, potential problems with using \([I]/K_i\) for prediction of in vivo interactions, especially for compounds that are highly protein-bound in plasma and microsomes. For these compounds, \([I]/K_{\text{Liu}}\), which corrects for the amount of free drug available in plasma and also normalizes the microsomal inhibition constant for the amount of free drug available in microsomes, is accepted as a more accurate predictor of a drug’s interaction.
potential to cause an in vivo interaction.\textsuperscript{35,36} In comparing the two ratios, [I]/\(K_I\) appears to underpredict the potential of DDEA to cause an interaction while greatly overpredicting the potential in vivo interaction of ODAA (the latter prediction changes from likely to remote when [I]/\(K_{int}\) is assessed instead of [I]/\(K_I\)). By application of either of the predictive methods, MDEA emerges as a possible contributor, whereas AMIO, DAA, and 3’-OHMDEA would be judged as unlikely to contribute to the potentiation of the warfarin anticoagulant effect. Also, although warfarin binds very strongly to plasma (\(f_{u(\text{serum})} = 0.008\)),\textsuperscript{37} it was unable to displace AMIO or its metabolites from plasma protein when incubated at a physiologic concentration, showing essentially no effect on \(f_u\) for any of the compounds tested.

The overall change in intrinsic clearance of (S)-warfarin caused by the drug interaction with AMIO can be predicted according to Eq. 1:

\[
\frac{\text{CL}_{int}}{\text{CL}_{int,\text{inhibited}}} = \frac{\text{AUC}_{\text{inhibited}}}{\text{AUC}} = 1 + \sum \frac{[I]}{K_I}
\]  

A sixfold increase in area under the plasma concentration–time curve (AUC) is therefore predicted for (S)-warfarin clearance based on the sum of the [I]/\(K_I\) ratios of the parent drug and circulating metabolites (Table 2). Serendipitously, if [I]/\(K_{int}\) ratios are used instead of [I]/\(K_I\), the same sixfold increase in AUC is predicted; however, the prediction arrived at using [I]/\(K_I\) is dominated by the ODAA ratio, whereas the prediction based on [I]/\(K_{int}\) values is driven almost entirely by DDEA (Table 2).

The actual increase in the mean AUC for (S)-warfarin caused by the drug interaction with AMIO has been reported to range from 27 to 110% in single warfarin dose studies carried out in small groups (\(n = 5\) or 6) of healthy volunteers.\textsuperscript{17,18} The data from our retrospective clinical evaluation, showing that introduction/discontinuation of AMIO therapy results in a 6–65% change in warfarin dose requirement, is in good agreement with these clearance data. However, it is also clear that our in vitro data overpredict the inhibitory effect of the overall drug interaction. Tertiary amines are increasingly recognized as being mechanism-based inactivators of P450 after undergoing sequential metabolism to C-nitroso metabolites whose nitrogen lone pair binds tightly to the reduced heme.\textsuperscript{38} Given that AMIO and its aminated metabolites are mixed inhibitors of CYP2C9, it is possible that one or more of these compounds could be a mechanism-based inhibitor of the enzyme. In fact, Mori et al. (2009) found AMIO and MDEA to be (weak) mechanism-based inhibitors of CYP2C9-mediated diclofenac 4’-hydroxylation, both exhibiting \(K_I\) values of inactivation of \(~100\mu\text{mol/l}\) and rate constants for inactivation (\(k_{\text{inact}}\)) values of only \(~0.1/\text{min}\).\textsuperscript{39} However, because we conducted our kinetic inhibition experiments with AMIO and MDEA at much lower concentrations than those indicated by the \(K_I\) values reported by Mori et al. (with the one exception of AMIO itself, for which we used a maximum concentration approximately equal to the reported \(K_I\) in the Mori study), it seems unlikely that time-dependent inhibition of microsomal CYP2C9 is a complicating feature of the kinetic plots obtained under the conditions used here, for either the parent drug or its primary metabolite. Nonetheless, studies are under way to evaluate the potential of each of the AMIO metabolites described here to evoke time-dependent P450 inhibition. It is possible that the discrepancy in our prediction of the DDI may be explained by a slow rate of membrane permeation coupled with high levels of drug/metabolite efflux resulting in a lower free drug concentration within the hepatocyte compared to plasma. The recent findings of Deng and co-workers, identifying all the known circulating AMIO metabolites (as well as a host of new metabolites) in human bile, lends support to this hypothesis.\textsuperscript{31}

European Medicines Agency guidelines currently recommend that only metabolites that circulate at unbound or total molar concentrations \(\geq 20\%\) of the parent drug be tested as potential causes of DDIs.\textsuperscript{40} Based on our results, DDEA appears to be the most likely metabolite to potentiate the anticoagulant effect when AMIO and warfarin are administered in combination, even though AMIO plasma concentrations were roughly 20-fold higher than DDEA concentrations in the patients studied. From our data it would appear that, at least in the specific case of drugs that contain secondary or tertiary amines, the current prediction system might be improved by the inclusion of mono- and (even more likely) di-\(N\)-desalkyl derivatives, even when these metabolites are found at very low concentrations in the circulation. This is because they are potentially much stronger P450 inhibitors than the parent drug. Studies are under way to determine whether patient variability in formation of DDEA, alone or in combination with other inhibitory metabolites, explains interindividual variability in the drug interaction between AMIO and warfarin.

METHODS
Retrospective clinical evaluation of the AMIO–warfarin drug interaction. From 1 July 2001 to 30 June 2007, a university-affiliated anticoagulation clinic provided routine outpatient anticoagulation management for 3,129 patients taking warfarin. During this 6-year period, a total of 230 incidents were identified in which out-of-range INR values were attributed to warfarin–AMIO drug interaction. These incidents were associated with 163 episodes in which amiodarone was added or discontinued in patients taking warfarin or in which the AMIO dose was changed. Of these 163 episodes, 90 were excluded from analysis for various reasons: unstable warfarin therapy before addition of AMIO (\(n = 40\)), an interaction due to change in AMIO dose (\(n = 27\)), warfarin and AMIO having been initiated concurrently (\(n = 14\)), the duration of concurrent warfarin/AMIO therapy being \(<1\text{ month (}\(n = 6\), or no records being available (\(n = 3\)). The 73 patients remaining in the cohort were mainly white (\(n = 66\)), with blacks (\(n = 3\)) and Asians (\(n = 4\)) also represented.

Recruitment of warfarin-treated patients for analysis of AMIO plasma metabolites. Three patients who had been stabilized on warfarin therapy and who were scheduled to be initiated into concomitant AMIO therapy were recruited through the Cardiology Division and the Anticoagulation Clinic at the University of Washington Medical Center. Briefly, blood was drawn at regular clinic visits for measurement of INR immediately before the addition of AMIO to the treatment regimen and at various time points during the subsequent 14-week period. Introduction of AMIO in the three patients resulted in a 33, 40, or 71% reduction in the required warfarin dose. All patients gave informed consent for the study, which was approved by the University of Washington institutional review board.

AMIO metabolite quantitative LC-MS assay. LC-MS analyses were conducted using a Micromass Quattro Premier XE Tandem Quadrupole Mass Spectrometer (Micromass, Manchester, UK) coupled to an
ACQUTY Ultra Performance LC (UPLC) System with integral autoinj ector (Waters, Milford, MA). The Premier XE was run in ESI–MS/MS MRM mode at a source temperature of 100°C and a desolvation temperature of 34°C. The cone voltage was set at 25 volts and the collision energy was set to 40 volts. The following mass transitions were monitored in separate ion channels: m/z 547 to 373 (for d₄-ODAA), 549 to 375 (d₂-ODAA), 590 to 547 (d₆-DDEA), 594 to 547 (d₈-DDEA), 591 to 547 (d₄-DAA), 593 to 549 (d₅-DAA), 618 to 547 (d₉-MDEA), 622 to 547 (d₄-MDEA), 646 to 547 (d₅-AMIO), 648 to 549 (d₆-AMIO), and 634 to 545 (3′-OH-DMEA). The parent drug and its metabolites were separated using only a Nucleosil 7.5 × 4.6 mm 5μ C₁₈-guard cartridge as column (Alltech, Deerfield, IL), with a flow rate of 0.35 ml/min, eluted with a binary solvent system consisting of 5 mmol/l NH₄OAc (solvent A) and 0.5% formic acid in MeOH (solvent B). The solvent was set at 50% B for 2 min and increased linearly to 100% B over the following 3.5 min. From 5.5 to 6.5 min, the solvent composition was lowered to 40% B, where it was maintained for 1 min (Figure 1).

Data analyses were carried out on Windows XP–based Micromass MassLynxNT software, version 4.1 (http://www.micromass-masslynx.software.informer.com).

Chemical inhibition experiments. Incubations were carried out using a pool of HLMs consisting of equal quantities of total microsomal protein obtained from eight different liver samples, prepared as described previously. All incubation mixtures contained 250 μg/ml microsomal protein from the HLM pool, 1 mmol/l NADPH, 1% vol/vol of a 100× substrate (at 1, 4, or 16 μmol/l final concentration), made up to 200 μl into polycarbonate ultracentrifugation tubes (Beckman-Coulter, cat. no. 547 (d₄-MDEA), 548 to 547 (d₂-AMIO), and 591 to 547 (3′-OH-DMEA). The parent drug and its metabolites were separated using only a Nucleosil 7.5 × 4.6 mm 5μ C₁₈-guard cartridge as column (Alltech, Deerfield, IL), with a flow rate of 0.35 ml/min, eluted with a binary solvent system consisting of 5 mmol/l NH₄OAc (solvent A) and 0.5% formic acid in MeOH (solvent B). The solvent was set at 50% B for 2 min and increased linearly to 100% B over the following 3.5 min. From 5.5 to 6.5 min, the solvent composition was lowered to 40% B, where it was maintained for 1 min (Figure 1).

Data analyses were carried out on Windows XP–based Micromass MassLynxNT software, version 4.1 (http://www.micromass-masslynx.software.informer.com).

HPLC-fluorescence assay for microsomal cytochrome CYP2C9 activity. HPLC was performed on a Shimadzu system equipped with two LC10ADvp pumps, a CBM-20A communication bus module, an RF-10AXL fluorescence detector, and an SIL-20AHT autosampler (Shimadzu Scientific Instruments, Columbia, MD) using a Nucleosil 5μ, 4.6 × 100 mm C₁₈, HPLC column (Machery-Nagel, Bethlehem, PA), with a flow rate of 1.3 ml/min. The analytical method was isocratic, set at 38% solvent B (acetonitrile (MeCN), solvent A) and 62% phosphoric acid) with a run time of 10 min. The fluorescent excitation wavelength was set at 320 nm and the emission was monitored at 415 nm. Data acquisition and analyses were performed using LC Solvents software (version 1.2; Shimadzu).

Determination of fᵢᵣ in HLMs and in plasma. Protein binding of AMIO and its metabolites was measured in both human plasma and pooled HLMs by ultracentrifugation using a TLA-100 benchtop ultracentrifuge with a TLA-100 rotor (Beckman-Coulter, Palo Alto, CA). AMIO or one of its metabolites was spiked (100× concentrated methanol stocks) into samples of either HLMs (0.25 mg/ml microsomal protein in 100 mmol/l KPi buffer, pH 7.4) or blank human plasma. Aliquots of 200 μl were taken from the samples and placed into polycarbonate ultracentrifugation tubes (Beckman-Coulter, cat. no. 3437775), which were either centrifuged at 100,000 rpm at 37°C for 2 h or incubated without centrifugation at the same temperature for the same time span. After ultracentrifugation, a 50-μl aliquot was removed from the clear, top layer of the plasma or the HLM supernatant and added to the same volume of MeCN. Similarly, after remixing, 50-μl aliquots were taken from the samples that had not been ultracentrifuged and added to MeCN. A standard solution (2.5 μl), containing a mix of d₁-AMIO, d₂-MDEA, d₅-DDEA, d₆-ODAA, and d₈-DAA (80 ng, 80 ng, 2.5 ng, 2.5 ng, and 2.5 ng, respectively) in methanol was added to each sample as internal standard. The samples were vortexed and centrifuged (13,400 rpm, 10 min), and the supernatants were transferred to vials for LC-MS analysis. All the reactions were carried out in triplicate. Standard curves were determined for both centrifuged and uncentrifuged samples over a range from 0.010 to 20 μmol/l. The free drug fractions in HLMs and plasma were calculated as the ratios of the respective slopes of the free drug concentration curves (determined from the ultracentrifuged samples) to the slopes of the total drug concentration curves (uncentrifuged samples).

Protocols for the synthesis of deuterium-labeled standards and for the quantitation of AMIO and its metabolites in plasma samples are provided in the Supplementary Materials and Methods online.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

ACKNOWLEDGMENTS

We thank Nina Isberrnan (University of Washington, Seattle) for helpful discussions and Sandy Harris, research nurse coordinator in the Division of Cardiology at the University of Washington School of Medicine, for her help in providing us with blood samples from patients undergoing warfarin/ amiodarone therapy. This work was supported by the National Institutes of Health (Project Program Grant P01 GM32165) to A.E.R.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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VOLUME 91 NUMBER 4 | APRIL 2012 | www.nature.com/cpt
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