JPET Fast Forward. Published on August 14, 2012 as DOI: 10.1124/jpet.112.196980

JPET #196980

Synthesis and antifungal activity of derivatives of 2- and 3-benzofurancarboxylic acids

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JPET Fast Forward. Published on August 14, 2012 as DOI: 10.1124/jpet.112.196980
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Running title is:

Synthesis and antifungal activity of benzofuran derivatives

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The number of

Text pages: 20

Tables: 2

Figures: 6

References: 51

Words in the Abstract: 218

Words in the Introduction: 726

Words in the Discussion: 1330

Recommended section assignment is Chemotherapy, Antibiotics, and Gene Therapy**.**

ABBREVIATIONS: IFI, invasive fungal infections; ICU, intensive care unit.

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ABSTRACT

We found that amiodarone has potent antifungal activity against a broad range of fungi potentially defining a new class of antimycotics. Investigations into its molecular mechanisms showed amiodarone mobilized intracellular Ca^{2+}, which is thought to be an important antifungal characteristic of its fungicidal activity. Amiodarone is a synthetic drug based on the benzofuran ring system, which is contained in numerous compounds both synthetic and isolated from natural sources with antifungal activity. In order to define the structural components responsible for antifungal activity, we synthesized a series of benzofuran derivatives not yet described previously in publications and tested them for inhibition of growth of two pathogenic fungi *Cryptococcus neoformans* and *Aspergillus fumigatus* in order to find new compounds with antifungal activity. We found several derivatives that inhibited fungal growth, two of which had significant antifungal activity. Surprisingly, we found that calcium fluxes in cells treated with these derivatives did not correlate directly with their antifungal effects, however the derivatives did augment the amiodarone-elicited calcium flux into the cytoplasm. We conclude that antifungal activity of these new compounds include activities in addition to changes in the cytoplasmic calcium concentration. Analyses of these benzofuran derivatives suggest that certain structural features are important for antifungal activity. Antifungal activity drastically increased on converting methyl 7-acetyl-6-hydroxy-3-methyl-2-benzofurancarboxylate (**2b**) into its dibromo derivative - methyl 7-acetyl-5-bromo-6-hydroxy-3-bromomethyl-2-benzofurancarboxylate(**4**).

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Introduction

The incidence of invasive fungal infections (IFIs) has risen dramatically due to an increase in the number of people with AIDS, undergoing bone-marrow and solid organ transplantations, high-dose chemotherapy, steroid treatment, and invasive medical procedures (Pfaller and Diekema, 2010; Pagano et al., 2011; Brissaud et al., 2012) and the increased use of immune suppression drugs has increased susceptibility to IFIs (Agrawal et al., 2011; Person et al., 2011). The familiar pathogens *Candida*, *Aspergillus*, and *Cryptococcus*, remain significant clinical problems. Fungal resistance to some antifungals has increased in recent years (Pfaller and Diekema, 2010). Hence there is need to develop alternative antimycotics, in particular those with novel mechanisms.

Cancer patients are particularly vulnerable to IFIs, which represent a major cause of morbidity and mortality (Sipsas and Kontoyiannis, 2012). *Aspergillus* is one of the main causes of IFIs in the ICU, causing 25% morbidity and 70% mortality (Andes et al., 2012). Invasive aspergillosis primarily affects immunocompromised individuals (Walsh and Stevens, 2011), but is now recognized as an emerging infection in ICU patients (Webb and Vikram, 2010; Stevens and Melikian, 2011).

Cryptococcosis due to *Cryptococcus neoformans* occurs worldwide and is one of the common complications in HIV/AIDS patients, organ transplant recipients, and patients with hematologic malignancies (Person et al., 2011). *Cryptococcus gattii* causes disease in healthy, immunocompetent persons as well as in immune compromised patients (Chaturvedi et al., 2005) and has infected hundreds of patients in recent outbreaks (Datta et al., 2009; Chaturvedi and Chaturvedi, 2011). *Cryptococcus* is thought to cause perhaps a million cases of cryptococcosis and 600,000 deaths per year worldwide (Park et al., 2009). Despite current antifungal therapy, mortality remains significant, between 10 and 25%, in AIDS patients (Li and Mody, 2010).

Amiodarone was found to have potent antifungal activity against a broad range of fungi, including *Cryptococcus*, *Aspergillus*, and *Candida*, potentially defining a new class of antimycotics (Courchesne, 2002). Amiodarone activates Ca^{2+} flux from both extracellular and intracellular stores (Courchesne and Ozturk, 2003). This Ca^{2+} flux is a major cause of its antifungal activity (Gupta et al., 2003; Muend and Rao, 2008; Zhang and Rao, 2008).

Amiodarone also induces stress responses that are modulated by components of the cell wall (Courchesne et al., 2009), which is considered to be a prime target for new antifungals.

Amiodarone is based on the benzo[b]furan ring system (Fig. 1), which is found in other synthetic and natural compounds that show antimicrobial activity. Egonol (Fig.1) and its synthetic derivatives are active against *Staphylococcus aureus, Bacillus subtilis, Candida albicans and Escherichia coli* (Emirdag-Ozturk et al., 2011). Notably, benzofurans reported by Masabuchi *et al.* (Masubuchi et al., 2001; Masubuchi et al., 2003) are *O*-alkylamino derivatives of 2-benzofurancarboxylic acids and amides and have antifungal activity. Furthermore, it was shown that esters and amides of substituted 2-benzofurancarboxylic acids, for example the compound RO-09-4609 (Fig.1), may act as inhibitors of fungal *N*-myristoyltransferase, thereby giving them antifungal activity (Masubuchi et al., 2001; Ebiike et al., 2002; Georgopapadakou, 2002; Kawasaki et al., 2003; Masubuchi et al., 2003). Benzofurans substituted at C-5 with OH have very recently been shown to have antifungal activity (Ryu et al., 2010). Finally, research by Kossakowski *et al*. (Kossakowski et al., 2010) presents data on halogenated derivatives of 3-benzofurancarboxylic acids. Methyl esters of 4-bromo-6-(dibromoacetyl)-5-hydroxy-2-methyl-1-benzofuran-3-carboxylic acid (brominated ester, Fig.1) and 4-chloro-6-(dichloroacetyl)-5-hydroxy-2-methyl-1-benzofuran-3-carboxylic acid exhibited antifungal activity against the *C. albicans* and *C. parapsilosis.*

These heterocyclic compounds show a variety of pharmacological properties and change of their structure offers a high degree of diversity that has proven useful in the search for new therapeutic agents, including many with antimicrobial activity (De Luca et al., 2009; Jiang et al., 2011; Kamal et al., 2011; Panchabhai et al., 2011).

To further our understanding of the molecular mechanism for amiodarone's antifungal activity we designed the syntheses of a series of substituted benzofurans with the goal of identifying structural components that account for its antifungal activity. Such compounds will help define the effective structures of amiodarone and potentially lead to new antifungal compounds better suited to clinical use than amiodarone. We found several new derivatives that inhibited fungal growth, two of which displayed significant antifungal activity. Calcium fluxes in cells treated with these derivatives did not correlate directly with their antifungal effects, nonetheless, these compounds potentiated the fungicidal activity or calcium mobilization caused

by amiodarone. Analyses of these benzofuran derivatives suggest that certain structural features are important for antifungal activity.

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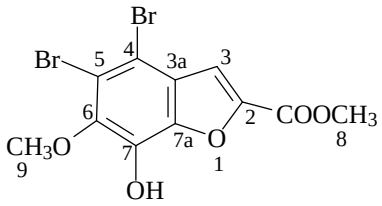
Materials and Methods

Synthesis of benzofuran derivatives. Melting points were determined with ElectroThermal 9001 Digital Melting Point apparatus and are uncorrected. Microanalysis was carried in the Department of Analytical Chemistry, Warsaw Technical University using Vario EL III, Elementar GmbH. High resolution mass spectra were recorded on Quattro LCT (TOF). ^{1}H NMR, ^{13}C NMR, HSQC and HMBC spectra in solution were recorded at 25 °C with a Varian NMRS-300 spectrometer. Chemical shifts δ [ppm] were referenced to TMS. IR spectra were recorded on a FT IR Spectrum 2000 Perkin Elmer instrument. TLC was carried out using Kieselgel 60 F_{254} sheets and spots were visualized by UV – 254 and 365 nm.

Methyl 4,5-dibromo-7-hydroxy-6-methoxy-2-benzofurancarboxylate (2e)



To the solution of methyl 5-bromo-7-hydroxy-6-methoxy-2-benzofurancarboxylate **(2d)** (0.04 mol) in glacial acetic acid (20 mL) bromine (0.675 g, 0.00425 mol) dissolved in acetic acid (5 mL) was added dropwise with stirring. The reaction mixture was stirred at ambient temperature for 2h, and then the precipitated oil was separated and purified by column chromatography on silica gel 230-400 mesh, eluent: $CHCl_3$.

Yield 51 %, solidified oil.

IR (KBr) cm^{-1}: 3466 (ν_{OH}), 3050 ($\nu_{C\text{-}Harom}$), 2952 ($\nu_{C\text{-}Hasym}$), 2845 ($\nu_{C\text{-}Hsym}$), 1703 ($\nu_{C=O}$), 1583, 1507, 1442 ($\nu_{C=C}$), 1442, 1435 (δ_{OH}), 1311 ($\nu_{C\text{-}O\text{-}Casym}$), 1279 ($\nu_{C\text{-}O\text{-}Casym}$), 1116 ($\nu_{C\text{-}O\text{-}Csym}$), 977, 886, 850, 829, 759 ($\gamma_{C\text{-}H}$);

^{1}H NMR (200 MHz, $CDCl_3$) δ = 4.05 (m, 6H, H-8, H-9), 7.50 (s, 1H, H-3).

Anal. $C_{11}H_8Br_2O_5$ (379.99) C H Br.

Methyl 7-acetyl-5-bromo-6-hydroxy-3-bromomethyl-2-benzofurancarboxylate (**4**)

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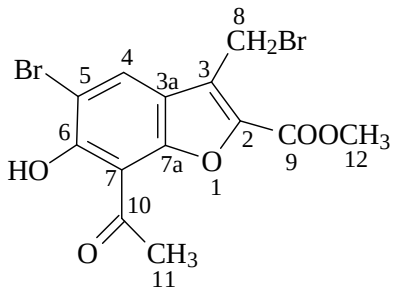

Methyl 7-acetyl-6-hydroxy-3-methyl-2-benzofurancarboxylate (**2b**) (1.98 g, 0.008 mol) was dissolved in glacial acetic acid (10 mL) at ambient temperature. Br_2 (1.05 mL, 3.2 g, 0.020 mol) dissolved in acetic acid (10 mL) was added dropwise with stirring for 1 h. The reaction mixture was stirred at ambient temperature for 6 h, then water (50 mL) and saturated $Na_2S_2O_3$ solution was added portion wise to remove any excess Br_2. Then the reaction mixture was extracted with $CHCl_3$ (3 x 60 mL), the organic layer was dried ($MgSO_4$ anh.). The solvent was evaporated and the residue crystallized (CH_3COOH). Yield 40 %, mp 167-168 ^oC.

IR (KBr) cm^{-1}: 3418 (ν_{OH}), 3064 ($\nu_{C\text{-}Harom}$), 2954, 2925 ($\nu_{C\text{-}Hasym}$), 2851 ($\nu_{C\text{-}Hsym}$), 1715 ($\nu_{C=O}$), 1628, 1603 ($\nu_{C=C}$), 1471, 1437 ($\delta_{C\text{-}Hasym}$), 1385,1342 (δ_{OH}), 1227, 1216 ($\nu_{C\text{-}O\text{-}Casym}$), 1052 ($\nu_{C\text{-}O\text{-}Csym}$), 930, 849, 823, 776 ($\gamma_{C\text{-}H}$);

^1H NMR (300 MHz, $CDCl_3$) δ = 2.98 (s, 3H, H-11), 4.02 (s, 3H, H-12), 4.94 (s, 2H, H-8), 8.17 (s, 1H, H-4), 13.98 (s, 1H, OH).

^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 19.61 (C8), 31.83 (C11), 52.84 (C12), 107.35 (C7), 109.71 (C5), 120.07 (C3a), 125.30 (C3), 131.47 (C4), 140.88 (C2), 153.06 (C7a), 159.51 (C9), 161.21 (C6), 202.49 (C10).

Anal. $C_{13}H_{10}Br_2O_5$ (406.03) C H Br.

TOF MS ES+: $[M+Na]^+$ calcd for $C_{13}H_{10}NaBr_2O_5$: 426.8803 found 426.8783.

Strains, media, and reagents. The *Cryptococcus neoformans* strain used was JEC21 (*MAT*α). The *Saccharomyces cerevisiae* strain used was FY70 (*MAT**a** ade1 trp1 leu2 his3 ura3*). The human leukemia cell line, K-562, is from the ATCC (CCL-243).

Cells were grown in SD (0.17 % w/v Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.4 % w/v ammonium sulfate, 2 % w/v glucose). Agar media contained 2 % w/v Bacto-agar. Adenine (12 mg/L final concentration), uridine (40 mg/L), leucine (30 mg/L),

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histidine (20 mg/L), and tryptophan (20 mg/L) were added to supplement auxotrophies as needed.

For in vitro studies, amiodarone (ICN) was added from a 10 mM stock in dimethylsulfoxide (DMSO). DMSO was added to the no drug cultures as a control. No effect on cell proliferation was observed by the addition of DMSO at the concentrations used (data not shown).

Yeast growth rates and viability. For quantification of *Cryptococcus neoformans* proliferation, cells were grown in 5 mL of liquid medium in Klett test tubes with vigorous shaking in a water bath. Measurement of cell density was done in a Klett-Summerson colorimeter. Cells were grown overnight in the medium to be tested. Dilutions of the overnight cultures were made into a series of test tubes containing fresh medium and various concentrations of drug or DMSO. The cell densities were monitored at time zero (typically about 1-2 x 10^6 cells/mL) and over time (typically 8-10 points for each growth curve) up to about 10 hours. The increase in cell density was plotted versus time and the resulting curves were used to determine the generation time for each culture (Courchesne, 2002). Each experiment was repeated 4-5 times and the mean values for generation times are given in the text and tables along with their standard errors.

For quantification of *Aspergillus fumigatus* proliferation, liquid cultures were inoculated with 10^5 conidia plus either 30 µl of DMSO only, 15 µM amiodarone (in DMSO), 25 µM **4** (in DMSO), or amiodarone plus **4.** *Aspergillus* cultures were then grown in minimal medium (5-20 mL) at 30°C with vigorous aeration for 4 days. Cultures were collected by filtration and the net dry weight of *Aspergillus* cells measured. The ratio of the weight of cultures treated with drug versus the weight of control cultures receiving only DMSO was calculated and presented as percent growth of drug-treated cultures in the text.

Human cell line growth rates. K-562 cells were grown in Iscove's modified Dulbecco's medium in culture flasks kept at 37°C. Cells were treated with methyl 5-bromo-7-(*O*-ethyl-2'-diethylamino)-6-methoxy-2-benzofurancarboxylate **(3d)**, compound **4**, amiodarone, or the carrier, DMSO, as a control. The growth of the cells was followed over a 3-day period. Control cells increased in density about 5 fold during this time (Fig. 3A), while cells treated with 10 µM amiodarone increased slightly less than 3 fold (Fig. 3B). Strikingly, cells treated with 10 µM **3d**

did not grow (Fig. 3C) and appeared dead by trypan blue staining (data not shown). K-562 cells were killed by exposure to either 30 μM amiodarone or 30 μM **3d.**

Aequorin luminescence. Cytoplasmic calcium concentration ($[Ca^{2+}]_{cyt}$) was followed using aequorin as described previously (Courchesne and Ozturk, 2003). Briefly, cells expressing apoaequorin in the cytoplasm cells were exposed to 5.9 μM coelenterazine for 1 hour to allow the cofactor to diffuse into cells and bind the apoaequorin to generate the holoprotein, aequorin. Cells (2.5×10^6) were aliquoted to a microtiter plate for luminescence measurement in an EG&G Berthold luminometer using Winglow monitoring and analysis software. The luminescence of aliquoted cells prior to any treatment was measured as a control, then at 30 seconds wells were injected with amiodarone in a volume of buffer equal to that of the cells, resulting in 50 μM final amiodarone concentration.

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Results

Chemistry. Compounds: 7-Acetyl-6-methoxy-3-methyl-2-benzofurancarboxylic acid (**1a**), methyl 7-acetyl-6-methoxy-3-methyl-2-benzofurancarboxylate (**2a**), methyl 7-acetyl-6-hydroxy-3-methyl-2-benzofurancarboxylate **(2b),** methyl 7-acetyl-6-(*O*-ethyl-2'-diethylamino)-3-methyl-2-benzofurancarboxylate **(3b),** methyl 6-hydroxy-7-(*p*-methoxycinnamoyl)-3-methyl-2-benzofurancarboxylate (**2c),** methyl 6-(*O*-ethyl-2'-diethylamino)-7-(*p*-methoxycinnamoyl)-3-methyl-2-benzofurancarboxylate (**3c),** methyl 5-bromo-7-hydroxy-6-methoxy-2-benzofurancarboxylate **(2d),** methyl 5-bromo-7-(*O*-ethyl-2'-diethylamino)-6-methoxy-2-benzofurancarboxylate **(3d),** methyl 7-acetyl-6-(*O*-ethyl-2'-diethylamino)-5-methoxy-3-methyl-2-benzofurancarboxylate **(3f),** methyl 6-acetyl-5-(*O*-ethyl-2'-diethylamino)-2-methyl-3-benzofurancarboxylate **(3g)** were prepared according to previously reported procedures (Kossakowski et al., 2005). The *O*-ethyl-*N*,*N*-diethylamino dervatives **3b-3d, 3f** and **3g** were converted to their hydrochlorides to improve their solubility in polar solvents.

The analytical data were in agreement with those reported previously.

The novel methyl 4**,**5-dibromo-7-hydroxy-6-methoxy-2- benzofurancarboxylate **(2e)** was synthesized by bromination of the ester **2d** with Br_2 in acetic acid.

Methyl 7-acetyl-5-bromo-6-hydroxy-3-bromomethyl-2-benzofurancarboxylate (**4)** was obtained by the multistep synthesis. 8-Acetyl-7-hydroxy-4-methylcoumarin was reacted with bromine in acetic acid to give 8-acetyl-3-bromo-7-hydroxy-4-methylcoumarin. Compound was converted to 7-acetyl-6-hydroxy-3-methyl-2-benzofurancarboxylic acid (**1b**) by reaction with sodium hydroxide. The acid **1b** was esterifed with methanol to give its methyl ester **2b** (Kossakowski et al., 2005). Bromination of the compound **2b** with bromine in acetic acid at ambient temperature afforded the novel benzofuran derivative - methyl 7-acetyl-5-bromo-6-hydroxy-3-bromomethyl-2- benzofurancarboxylate (**4**) (Scheme 1). The structures are presented in Fig. 2.

In Vitro Antifungal Activity of Benzofuran Derivatives. We screened the benzofuran derivatives **1a, 2a-2e, 3b-3d, 3f, 3g** and **4** for antifungal activity. We tested whether these derivatives could inhibit the growth of the opportunistic pathogen *C. neoformans* and found effects ranging from nearly none to severe growth inhibition. Table 1 shows the generation times of *C. neoformans* strain JEC21 treated with these derivatives growing in minimal media in the presence of 20 µM benzofuran derivatives or just the carrier (DMSO) as a control. Cells treated

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with derivatives **1** and **3f** had generation times like the control cells treated with DMSO, while cells treated with derivatives **2a**, **2c**, **2d**, **2e**, **3c** and **3g**, and had generation times only about 10-20% slower than controls. Strikingly, cells treated with 20 µM **3d** and **4** arrested growth immediately upon addition. The viability of the growth-arrested cells was tested by the Live/Dead Lumofungin assay and the derivative-treated cells were found to be metabolically dead (data not shown). Because of the potent antifungal activity exhibited by **3d** and **4** we examined the effects these two derivatives in greater detail (Table 2).

JEC21 cells treated with 10 µM **3d** did not grow during the 8-10 hour period of the experiment, while cells treated with 5 and 2 µM **3d** had generation times 600% and 12% slower than controls, respectively (Table 2). Cells treated with 10 and 5 µM **4** had generation times 200% and 15% slower than controls.

Effects of 3d and 4 on Mammalian Cell Growth In Vitro. Since it is desirable to identify benzofuran derivatives that retain antifungal activity but have reduced toxicity toward mammalian cells we tested the effects of **3d** and **4** on the growth of the human leukemia cell line K-562.

K-562 cells were treated with **3d**, **4**, amiodarone, or the carrier, DMSO, as a control. The growth of the cells was followed over a 3-day period. Control cells treated with two amounts of DMSO increased in density about 5 fold during this time (Fig. 3A), while cells treated with 10 µM amiodarone increased slightly less than 3 fold (Fig. 3B). Strikingly, cells treated with 10 µM **3d** did not grow (Fig. 3C) and appeared dead by trypan blue staining (data not shown). K-562 cells were killed by exposure to either 30 µM amiodarone or 30 µM **3d**.

4 was less toxic to the K-562. Cells treated with both 10 µM and 30 µM **4** increased more than 4 fold during the period of the experiment, similar to the growth of the control (Fig. 3D). When **4** was added at 50 µM, cell growth was slowed slightly. **4** treated cells had a 54.2 hour (standard error of 10.4) generation time compared to 34.0 (2.5) for control cells, a 60% increase in the time required per cell generation.

Thus, amiodarone is moderately toxic to K-562 cells, while **3d** shows significantly more toxicity than amiodarone does. In contrast, **4** shows no effects on cell growth at concentrations (10 - 30 µM) that are completely inhibitory to yeast growth and shows only mild growth inhibition at a higher concentration.

3d and 4 Affect Yeast Cytoplasmic Ca^{2+}. We have shown that amiodarone causes a major rise in the cytoplasmic concentration of Ca^{2+} ($[Ca^{2+}]_{cyt}$) in *Saccharomyces cerevisiae* cells (Courchesne and Ozturk, 2003). We tested for similar effects on the $[Ca^{2+}]_{cyt}$ in *S. cerevisiae* by **3d** and **4** (Fig. 4). *S. cerevisiae* cells (FY70 [pEVP11/AEQ]) expressing apoaequorin were assayed for their response to amiodarone, **3d**, and **4**. Cells were treated with 50 µM amiodarone, **3d**, or **4** at time zero and the increase in RLUs/sec were monitored for 3.5 min. The increase in RLUs is proportional to the increase in the $[Ca^{2+}]_{cyt}$. Amiodarone elicited an immediate and very large increase in RLUs while **3d** elicited a much smaller increase, about 5 fold smaller than amiodarone (Fig. 4A and 4B, respectively). In contrast, **4** elicited no increase in the RLUs (Fig. 4C), producing a response like that of the control cells treated with carrier only (Fig. 4D). An inability to stimulate Ca^{2+} flux also occurred with each of the other derivatives shown in Table 1 (data not shown). Thus, the various benzofuran derivatives display distinct abilities to elicit increases in the $[Ca^{2+}]_{cyt}$ that do not correspond with their relative growth inhibitory effects.

We also investigated whether pretreatment of yeast cells with benzofuran derivatives could affect the calcium response elicited by subsequent amiodarone treatment (Fig. 5). *S. cerevisiae* cells were prepared for aequorin assays as just described. As a control, cells were pretreated with carrier only (DMSO) at zero time and then amiodarone was added at 30 sec. This resulted in the same increase in RLUs elicited by amiodarone addition with no pretreatment (Fig. 5A). Pretreatment with **2a** did not change the maximum level of RLU but shifted the response about 10 sec sooner (Fig. 5B). **3f** and **3g** pretreatment increased the maximum RLUs about 40% versus the control (Fig. 5C and 5D, respectively). **3d** increased the maximum RLUs by about 55% and shifted the response by about 10 sec sooner (Fig. 5E). In contrast, pretreatment with **4** increased the maximum RLUs about 100% and shifted the response about 20 sec earlier (Fig. 5F), demonstrating a significant stimulating effect by **4** on the rise in $[Ca^{2+}]_{cyt}$ caused by amiodarone.

4 Stimulates Amiodarone's Antifungal Activity. We then checked whether **4** also stimulated amiodarone's antifungal activity against the pathogenic fungi *Aspergillus fumigatus*. Growth of *Aspergillus* was measured by inoculating 10^5 spores into liquid minimal growth medium containing amiodarone only, **4** only, or amiodarone plus **4**. The control culture was treated with only the carrier DMSO. After 4 days of growth, the cultures were removed from the

liquid medium, dried and total culture weighed to measure the amount of growth. The ratios of the weight of drug-treated versus control cultures were calculated. The mean ratios and standard errors from three independent experiments were determined.

15 μM amiodarone treatment had no deleterious effect on growth of *Aspergillus*, possibly even increasing growth slightly to 113% (std. error = 18) of control. Cultures treated with 25 μM **4** produced 77% (std. error = 14) of the weight of control cultures, revealing a slight growth inhibition. In striking contrast, cultures treated with a combination of 25 μM **4** plus 15 μM amiodarone produced only 3.7% (std. error = 1.3) of the weight of the control culture, showing a marked growth inhibition, which was more than 20-fold lower than either drug by itself. These results show that **4** acts in concert with amiodarone to have a major inhibitory effect on growth of *Aspergillus*.

Discussion

Amiodarone is a potent antiarrythmic drug, which is used to treat all forms of supraventricular and ventricular tachycardia. Although widely used it is known to have significant adverse side effects on various organs, such as the liver, neuromuscular system, thyroid, cornea, skin, and lungs (for a review see Connolly, 1999). Although several organ systems can be affected, pulmonary complications are perhaps the most deleterious and occur in about 10% of patients, usually after two or more months of treatment (Kennedy et al., 1987) and clinically significant lung damage appears to require a very high total dose of amiodarone (as much as 200 gm (Dusman et al., 1990). Nonetheless, acute presentations can occur within weeks of initiating treatment (Ashrafian and Davey, 2001; Lardinois et al., 2002) and the problem of adverse effects is a limiting factor for long-term utilization of amiodarone. Benzofuran-based compounds related to amiodarone with improved safety profiles are being developed to replace amiodarone. For example, in 2009 the FDA approved dronedarone, a benzofuran-based compound closely related to amiodarone, for clinical use as an antiarrhythmic drug. In an analogous approach, the goal of our research was to identify novel benzofuran derivatives that retained amiodarone's antifungal activity but with reduced toxicity.

In order to exploit the potential antifungal activity of amiodarone and synthesize new derivatives better suited to clinical use, it is essential to identify the structures that function in this capacity. Amiodarone is a complex synthetic drug based on a benzofuran ring system, which is an important pharmacophore contained in numerous compounds that can be isolated from natural sources as well as in synthetic products. Many of these heterocyclic compounds are simpler in structure than amiodarone and have proven to be useful in the search for new therapeutic agents including many with antifungal activity (Masubuchi et al., 2003; Kossakowski et al., 2010; Ryu et al., 2010). Comparison of amiodarone and these other compounds strongly supports the hypothesis that biological activity and therapeutic application of amiodarone relies in particular on the pattern of substitution on the aromatic ring.

To investigate the role of the benzofuran ring system, in particular substitutions on the aromatic ring, we synthesized and screened a series of benzofuran derivatives for antifungal activity. Although several derivatives did not significantly inhibit yeast cell growth, cryptococcal cells treated with derivatives **2a**, **2b**, **2c**, **2d**, **2e**, **3c** and **3g**, were growth inhibited and had

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generation times ranging from 10-20% slower than control cells treated with DMSO. These are modest effects on fungal growth but demonstrate that different derivatives can have antifungal effects. The substitutions can now be evaluated for synthesis of future derivatives.

Importantly, we found two derivatives with significant antifungal activity. Derivatives **3d** and **4** had substantial fungicidal activity against *Cryptococcus neoformans*. **3d** showed inhibition of cryptococcal growth at 2 μM and growth was completely inhibited at 10 μM, while **4** required 5 μM to cause significant inhibition, while 20 μM prevented cryptococcal growth. Thus, **3d** possesses the more potent antifungal activity that is effective in about the same concentrations as is amiodarone. Although **4** has slightly less active antifungal activity on its own, it was found to have a major stimulating activity in combination with amiodarone. Importantly, **4** showed low toxicity to a human leukemia cell line, suggesting a potential for therapeutic use. Thus, **4** has the desired characteristics of a second-generation amiodarone derivative. We first saw stimulating activity when **4** was tested for its effects on the ability of amiodarone to mobilize Ca^{2+} in yeast cells. Yeast cells treated with both drugs increased their $[Ca^{2+}]_{cyt}$ to about twice the level, and more rapidly, as cells treated with amiodarone alone. **3d** Also showed such stimulation but to a significantly lesser extent than **4**.

We explored the molecular characteristics of **3d** and **4**, testing their abilities to elicit calcium fluxes in yeast cells, as amiodarone does. The ability to mobilize Ca^{2+} is thought to be an important antifungal characteristic of amiodarone (Courchesne and Ozturk, 2003; Gupta et al., 2003). Surprisingly, we found that calcium fluxes in cells treated with these derivatives were significantly different than for amiodarone and did not correlate with their antifungal effects. While the antifungal activity of **3d** was similar to amiodarone and **3d**'s toxicity to human cells even greater, its ability to elicit a calcium flux in yeast was about 4 fold lower than amiodarone. Although **4** demonstrated significant antifungal activity, it was ineffective, by itself, in promoting calcium flux in yeast, yet stimulated calcium flux by amiodarone. These results provide a basic understanding of the mechanism of action for the compounds studied here; they act similarly but not identically to amiodarone and we speculate their antifungal activity may include activities in addition to dramatic changes in the cytoplasmic calcium concentration.

In addition to its in vitro antifungal activity, amiodarone has shown antiprotozoal activity, by itself and in combination with certain antifungal drugs, in vitro (Benaim et al., 2006; Serrano-Martin et al., 2009a; de Macedo-Silva et al., 2011) and in vivo in murine models (Benaim et al.,

JPET Fast Forward. Published on August 14, 2012 as DOI: 10.1124/jpet.112.196980
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2006; Serrano-Martin et al., 2009b; Bobbala et al., 2010). Importantly, there have been clinical case reports showing amiodarone efficacy in humans against Chagas' disease (Paniz-Mondolfi et al., 2009) and cutaneous leishmaniasis (Paniz-Mondolfi et al., 2008). Given the lack of good alternatives, the authors speculated that amiodarone might be considered for clinical treatment of these parasites. In this regard, we have shown that our compound **4** dramatically stimulates amiodarone's antifungal activity to inhibit *Aspergillus*, thus we speculate that compound **4** would also stimulate amiodarone's antiparasitic activity as well. It should be noted that the recently approved amiodarone replacement drug, dronedarone has also been shown to have antiparasitic activity in vitro (Benaim et al., 2012), raising the possibility that it may be effective in vivo against these parasites and be a less toxic alternative to amiodarone. This hope may be premature even though dronedarone was FDA approved following reports of an improved safety profile (Hohnloser et al., 2009) because more recent studies cast doubt on its safety (Said et al., 2012). Should additional studies show dronedarone to truly be safer than amiodarone, particularly during short-term use against parasitic agents, it could, in theory, be tested for antifungal activity by itself and in combination with antifungal drugs, in particular our compound **4**.

Our findings suggest that certain structural features are important for antifungal activity. As indicated earlier, compounds **2a** and **2b** have modest effects on fungal growth. Changing the position of the acetyl substituent (at C-5 in the compound **2a** and C-7 in **2b**), and methylation the C-6 phenolic group as well as the modification of the C-7 acetyl group in the compound **2b** leading to **2c,** do not result in any significant effect towards the antifungal activity.

In view of the influence of bromine atoms in the aromatic ring on the antifungal activity we have shown that introducing additional bromine does not work - both benzofurans derivatives: monobrominated at C-5 compound **2d** and C-4 and C-5 *o*-dibrominated compound **2e** are modestly active.

The introduction of an ethyl-*N,N*-diethylamino group to moderately active compound **2d** resulted in improving its antifungal activity, as it was proved for **3d**. However, similar modification carried out in **2c** did not yield the active compound.

Double bromination (in the aromatic ring and C-3 methyl group) of the moderately active compound **2b** afforded the active compound **4.** Compounds, **3d** and **4** have similar substitution pattern on benzofuran moiety (drawn in bold, see Scheme 1) that may be responsible for antifungal activity. However, the lack of alkyl substituent on C-3 in **3d** may be associated with

high toxicity. Similarly, *4-methylcoumarin* is 3.5-8.5 times less *toxic* than coumarin (Feuer, 1974; Fernyhough et al., 1994) but 4-methylumbelliferone (7-hydroxy-4-methylcoumarin) is approved as food additive and a drug. We hypothesize that the key moiety, responsible for compound **4**'s antifungal activity is the CH_2Br substituent at C-3. It increases the lipophilicity and gives the compound **4** the opportunity to act as an alkylating agent. Future studies will investigate the role of this key moiety.

Authorship Contributions

Participated in research design: Courchesne, Hejchman

Conducted experiments: Courchesne, Ostrowska, Kossakowski

Performed data analysis: Courchesne, Hejchman, Maciejewska

Wrote or contributed to the writing of the manuscript: Courchesne, Hejchman, Maciejewska

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Figure legends

Scheme 1. Synthesis of methyl 5-bromo-7-(*O*-ethyl-2'-diethylamino)-6-methoxy-2-benzofurancarboxylate **(3d)** and methyl 7-acetyl-5-bromo-6-hydroxy-3-bromomethyl-2-benzofurancarboxylate (**4**).

Fig. 1. Benzofuran derivatives with antifungal activity.

Fig. 2. Structures of tested benzofuran derivatives.

Fig. 3. Growth of the human cell line K-562 when given (A) DMSO only, (B) 10 µM or 30 µM amiodarone (in 20 and 60 µl DMSO, respectively), (C) 10 µM or 30 µM **3d** (in 20 and 60 µl DMSO, respectively), and (D) 10 µM, 30 µM, and 50 µM **4** (in 20, 60, and 100 µl DMSO, respectively).

Fig. 4. Intracellular Ca^{2+} was monitored by measuring the relative light units (RLU) per second given off by aequorin after cells were exposed to (A) 50 µM amiodarone, (B) **3d**, (C) **4**, or (D) the ethanol (EtOH)/DMSO carrier only. Monitoring began at 0 seconds and compounds were added at 30 seconds and the response monitored for an additional 180 seconds.

Fig. 5. Intracellular Ca^{2+} was monitored as described in Fig. 4. In each case 50 µM amiodarone (amio.) was given to the cells along with 50 µM of the test compounds. (A) amiodarone only, (B) amio. + **3f**, (C) amio. + **2a**, (D) amio. + **4**, (E) amio. + **3g**, and (F) amio. + **3d**. The dotted curves in (B - F) are the mean response curve of amiodarone only from (A) to allow a direct comparison of the change in the response curve due to the added compound.

JPET Fast Forward. Published on August 14, 2012 as DOI: 10.1124/jpet.112.196980
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TABLE 1

Cells of *Cryptococcus neoformans* strain JEC21 were grown in minimal medium, 30°C, containing the indicated benzofuran derivative (20 µM added in 10 µl DMSO) or just the carrier (DMSO, 10 µl). The increases in cell number were measured over 6-10 hour periods. The generation times (given in minutes) are the means of 3-4 experiments and were determined by linear regression with reliability values of 0.9 or above. The standard errors are shown.

Derivative	Generation time	Standard error
DMSO	167	4.8
2a	184	6.6
2b	219	5.8
2c	189	14.4
2d	205	21.2
2e	194	7.8
3c	188	19.6
3g	180	6.8
3d	No Growth	---
4	No Growth	---

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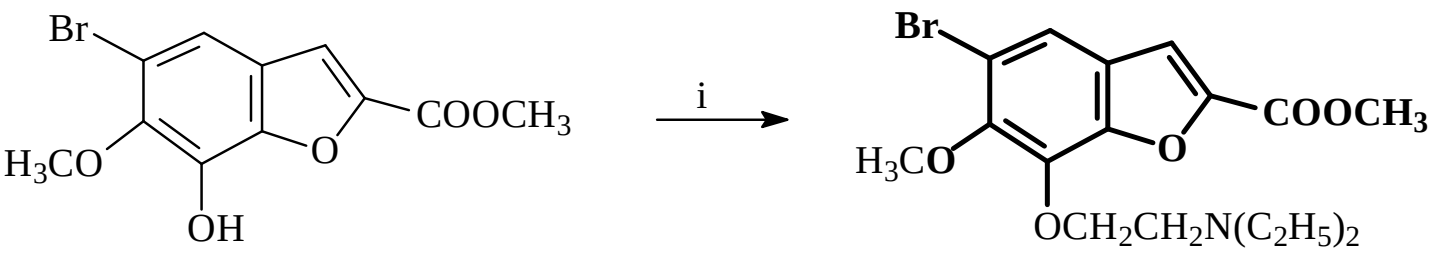
TABLE 2

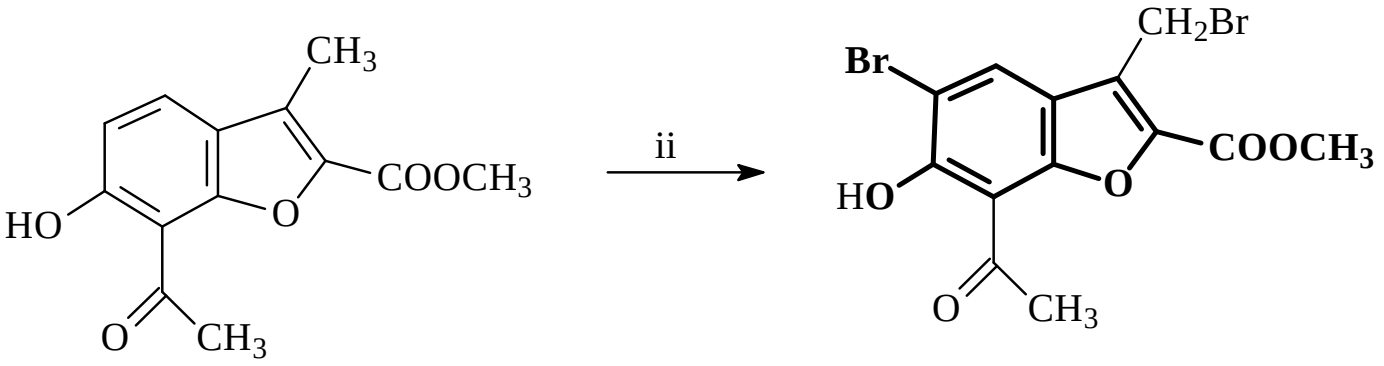
Cells of *Cryptococcus neoformans* strain JEC21 were grown in minimal medium, 30°C containing the indicated concentrations of **3d** or **4** (added in 1 µl, 2.5 µl, or 5 µl of DMSO) or DMSO only (1 µl, 2.5 µl, or 5 µl). The increases in cell numbers were measured over 6-10 hour periods. The generation times (given in minutes) were determined by linear regression with reliability values of 0.9 or above. The means plus standard errors (in parentheses) of three independent experiments are shown.

Derivative	2 µm	5 µM	10 µM
DMSO	189 (12)	186 (14)	182 (10)
3d	237 (28)	1064 (188)	No Growth
4	Not determined	276 (17)	395 (125)

Scheme 1



2d **3d**

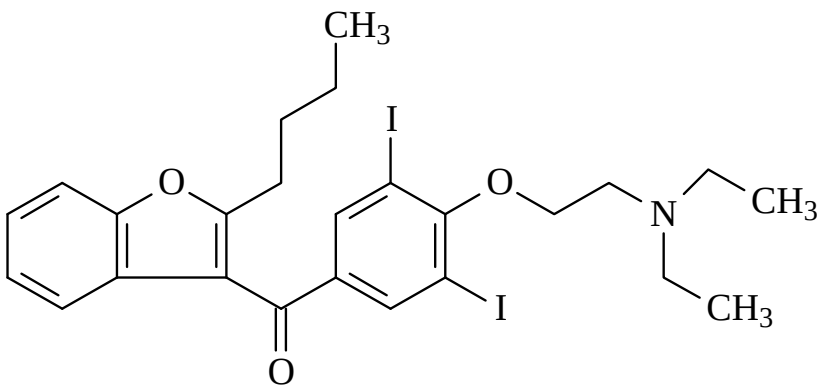


2b **4**

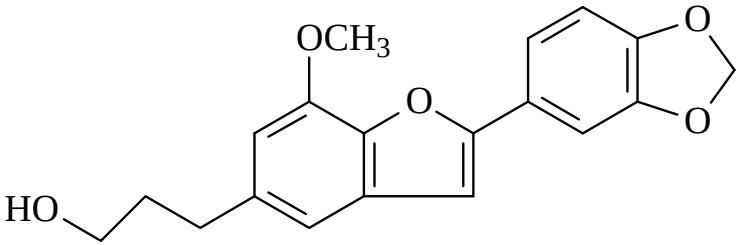
i: $Cl(CH_2)_2N(C_2H_5)_2 \cdot HCl$, K_2CO_3 anh, Aliquat 336, acetone, reflux

ii: Br_2 in AcOH, r.t.

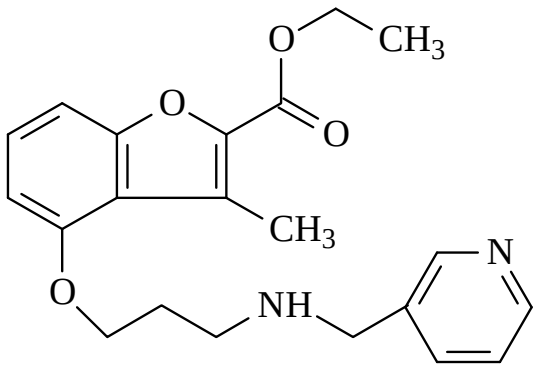
Fig. 1



Amiodarone



Egonol



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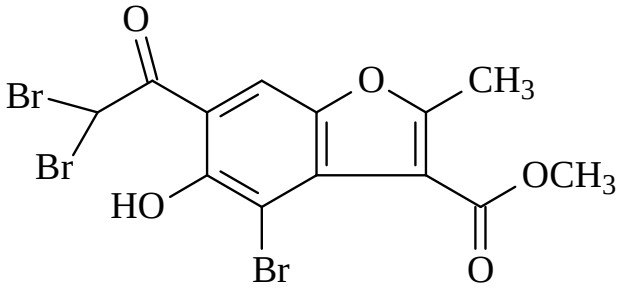
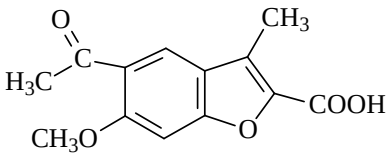
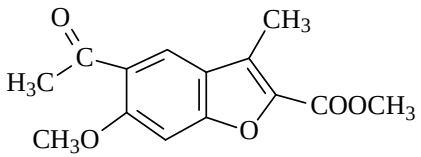


Brominated ester I

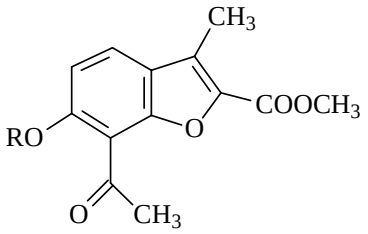
Fig. 2



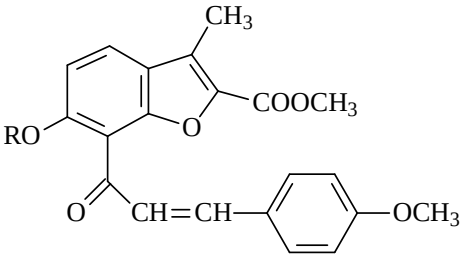
1a



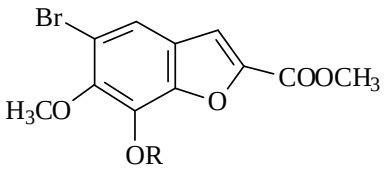
2a



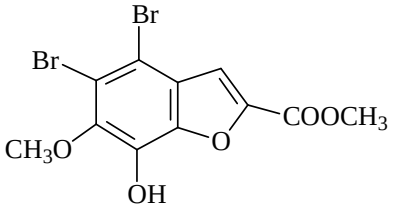
2b: R = H
3b: R = -$(CH_2)_2N(C_2H_5)_2$



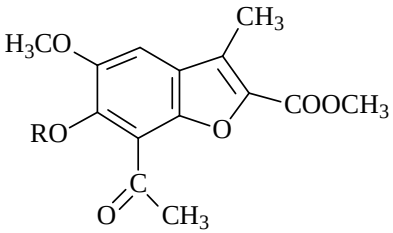
2c: R = H
3c: R = -$(CH_2)_2N(C_2H_5)_2$



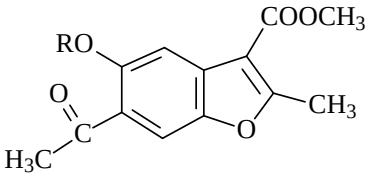
2d: R = H
3d: R = -$(CH_2)_2N(C_2H_5)_2$



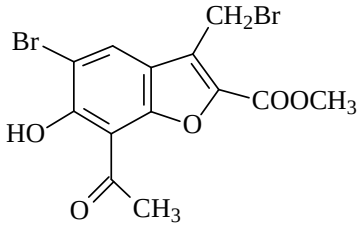
2e



3f: R = -$(CH_2)_2N(C_2H_5)_2$



3g: R = -$(CH_2)_2N(C_2H_5)_2$



4

Fig. 3

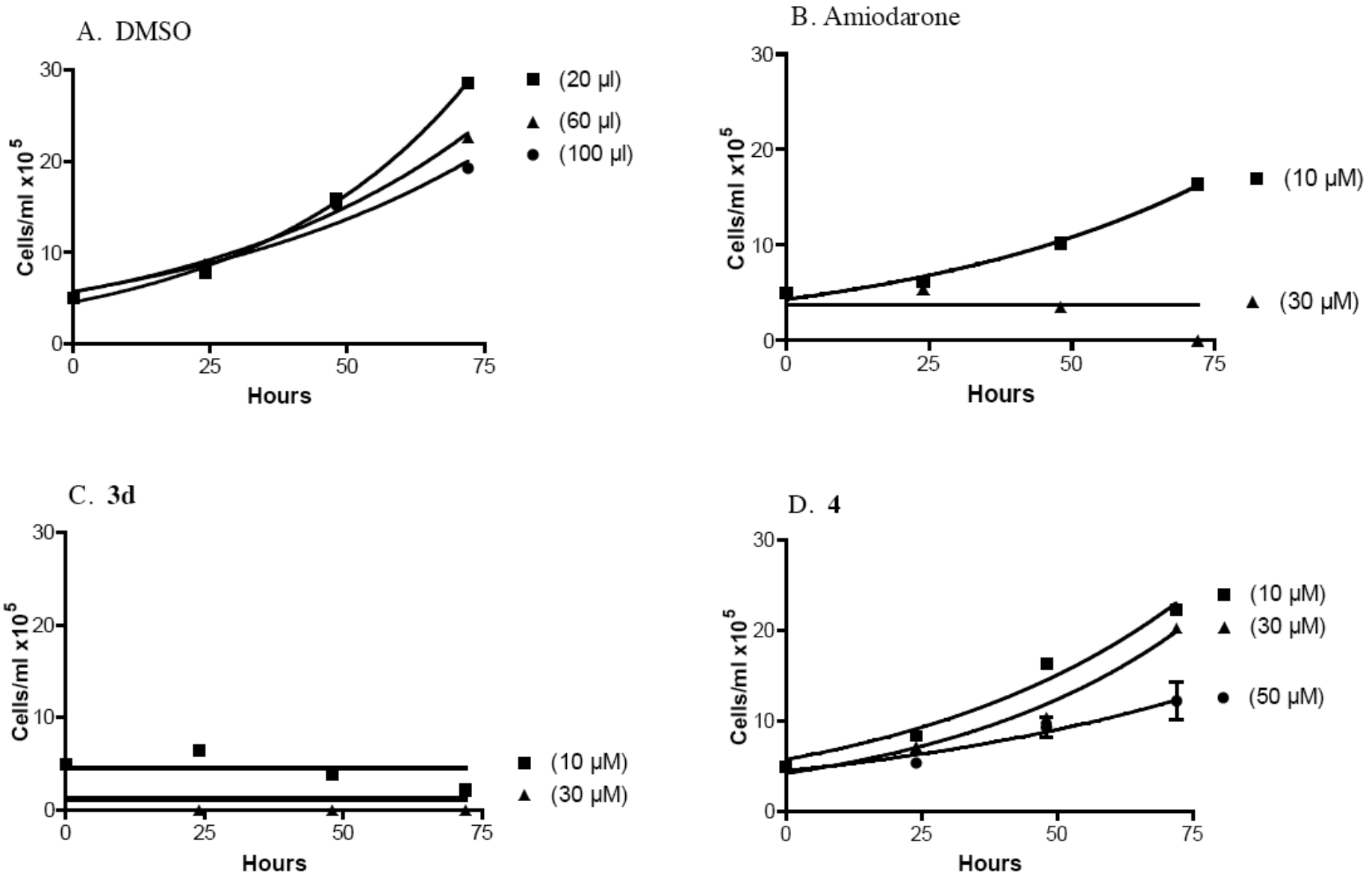
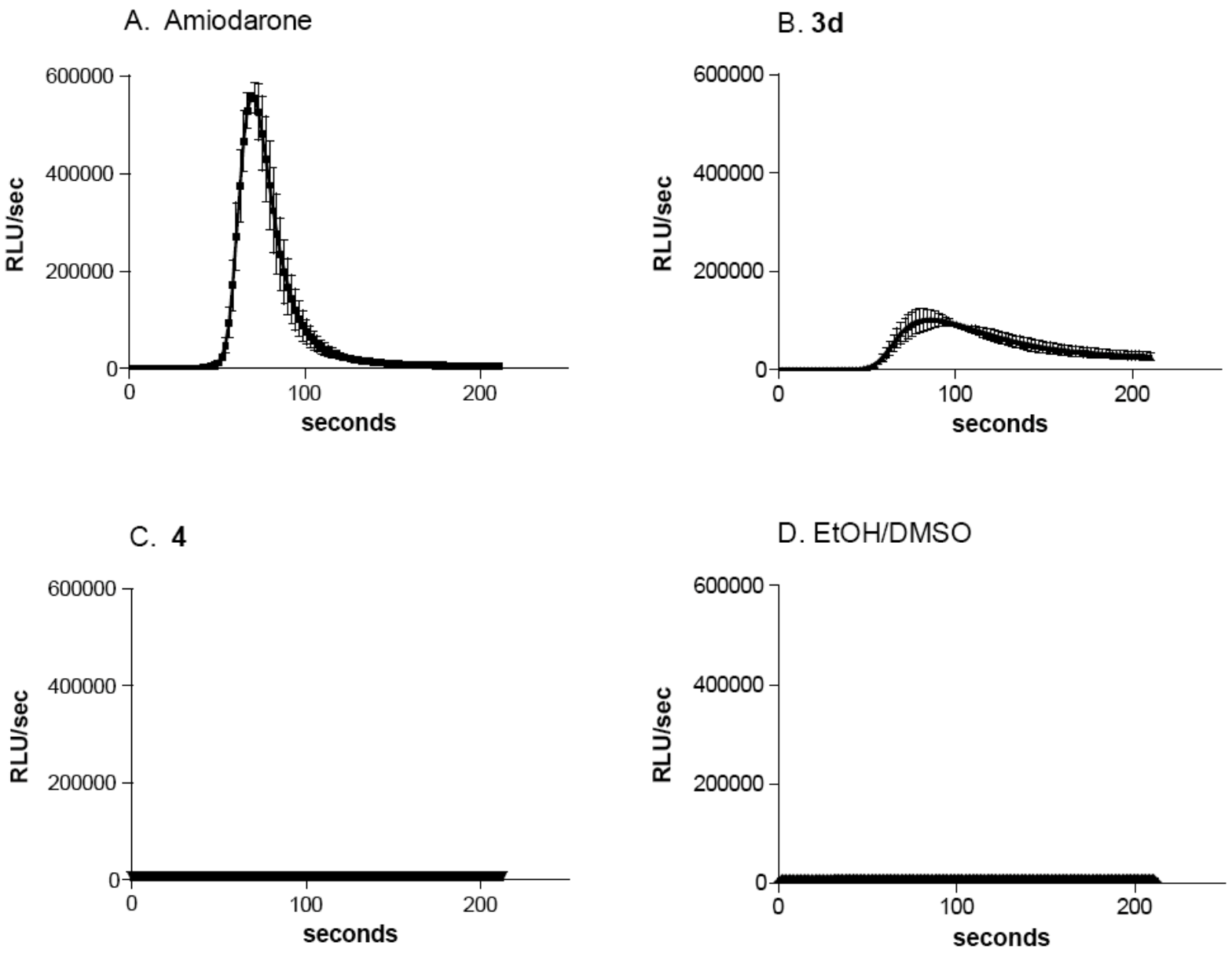


Fig. 4



JPET Fast Forward. Published on August 14, 2012 as DOI: 10.1124/jpet.112.196980
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Fig. 5