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Inhibition of xanthine oxidase by the aldehyde oxidase inhibitor raloxifene: Implications for identifying molybdopterin nitrite reductases

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Abstract

Sources of nitric oxide alternative to nitric oxide synthases are gaining significant traction as crucial mediators of vessel function under hypoxic inflammatory conditions. For example, capacity to catalyze the one electron reduction of nitrite (NO_2^-) to •NO has been reported for hemoglobin, myoglobin and molybdopterin-containing enzymes including xanthine oxidoreductase (XOR) and aldehyde oxidase (AO). For XOR and AO, use of selective inhibition strategies is therefore crucial when attempting to assign relative contributions to nitrite-mediated •NO formation in cells and tissue. To this end, XOR inhibition has been accomplished with application of classic pyrazolopyrimidine-based inhibitors allo/oxypurinol or the newly FDA-approved XOR-specific inhibitor, Uloric® (febuxostat). Likewise, raloxifene, an estrogen receptor antagonist, has been identified as a potent (K_i = 1.0 nM) inhibitor of AO. Herein, we characterize the inhibition kinetics of raloxifene for XOR and describe the resultant effects on inhibiting XO-catalyzed •NO formation. Exposure of purified XO to raloxifene (PBS, pH 7.4) resulted in a dose-dependent (12.5–100 μM) inhibition of xanthine oxidation to uric acid. Dixon plot analysis revealed a competitive inhibition process with a K_i = 13 μM. This inhibitory process was more effective under acidic pH; similar to values encountered under hypoxic/inflammatory conditions. In addition, raloxifene also inhibited anoxic XO-catalyzed reduction of NO_2^- to •NO (EC_{50} = 64 μM). In contrast to having no effect on XO-catalyzed uric acid production, the AO inhibitor menadione demonstrated potent inhibition of XO-catalyzed NO_2^- reduction (EC_{50} = 60 nM); somewhat similar to the XO-specific inhibitor, febuxostat (EC_{50} = 4 nM). Importantly, febuxostat was found to be a very poor inhibitor of human AO (EC_{50} = 613 μM) suggesting its usefulness for validating XO-dependent contributions to NO_2^- reduction in biological systems. Combined, these data indicate care should be taken when choosing inhibition strategies as well as inhibitor concentrations when assigning relative NO_2^- reductase activity of AO and XOR.

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Keywords

Aldehyde oxidase; Nitrite; Xanthine oxidoreductase; Raloxifene; Nitric oxide; Febuxostat

Introduction

Sources of nitric oxide alternative to the enzymatic activity nitric oxide synthases are currently being investigated as mediators of vascular function under hypoxic/inflammatory conditions. As a result, it has become apparent that inorganic nitrite (NO_2^-) can serve as a robust reservoir of •NO where hypoxia and acidic pH facilitate both non-enzymatic and enzymatic processes that reduce NO_2^- to •NO [1,2]. One of the critical enzymatic processes reported to perform this NO_2^- reductase activity has been assigned to the molybdopterin family of enzymes; more specifically xanthine oxidoreductase (XOR) and aldehyde oxidase AO (AO), although other family members are currently under investigation. Recent reports have demonstrated NO_2^- reductase activity for both XOR and AO where NO_2^- is reduced by one electron to •NO at the Mo-cofactor (Mo-co) when reducing equivalents are supplied directly to the Mo-co by hypo/xanthine (XOR) and/or aldehydes (AO) or indirectly via reduction of their respective FAD-cofactors by NADH [3–7]. This NO_2^- reductase activity is inhibited by O_2 and thus optimally operative under low O_2 tensions. Details regarding the micro-environmental factors influencing •NO production capacity from XOR and AO have been recently reviewed in this journal [8].

Several tissues express abundant XOR as well as AO activity including the liver, intestine and lung. Therefore, assigning relative contributions of XOR and AO to NO_2^- – dependent •NO formation necessitates either specific inhibition strategies or validation that the tissue in question does not express XOR or AO protein and/or activity. For the former, no commercially available antibodies exist that can distinguish between XOR and AO due to significant sequence homology (86%) between the two enzymes. For the later, both XOR and AO demonstrate a significant degree of promiscuity for substrates at their Mo-co active site. Adding to the frustration, XOR tissue-specific conditional knockouts are currently not available while global $XOR^{-/-}$ and $XOR^{+/-}$ mice experience alterations in nutrient absorption and elevated plasma hypoxanthine levels resulting in death from kidney failure before 6 weeks of age [9,10]. As for AO, there is only one report demonstrating successful knockout of one homologue of AO (aldehyde oxidase homologue 2, *Aoh2*) expressed primarily in the epithelium [11]. The current absence of knockout strategies to interrogate these molybdopterin enzymes has relegated investigators to employ pharmacologic means to conduct proof of principle experimentation regarding contributory roles mediating the effects of NO_2^- treatment. This being said, inhibitors of the Mo-co may also display overlap where-by an XOR inhibitor may partially inhibit AO or *vice versa*; especially when utilizing higher inhibitor concentrations. Recently, Uloric® (febuxostat) has been identified as a potent XOR-specific inhibitor (K_i = 0.96 nM) [12]. Likewise, the estrogen receptor antagonist, raloxifene has been distinguished as a potent AO inhibitor (K_i = 1.0 nM) [13]. These inhibitory characteristics have led investigators to use raloxifene and febuxostat to distinguish AO-dependent NO_2^- reduction from that mediated by XOR. While this approach

seems appropriate, the absence of cross-over inhibition analysis with both enzymes is problematic. Herein, we characterize the inhibition properties of raloxifene for XO and febuxostat for AO in order to more clearly define an approach with optimal potential for success.

Materials and methods

Materials

Xanthine, raloxifene, allopurinol, sodium nitrite, and menadione were from Sigma (USA). Xanthine oxidase (XO) was from Calbiochem (USA). Heparin Sepharose 6B Fast Flow (HS6B) was purchased from GE Healthcare (USA). Febuxostat was purchased from BIOTANG (USA). The •NO donor 1-(hydroxy-NNO-azoxy)-*L*-proline (PROLI NONOate) and the electron paramagnetic resonance (EPR) spin trap 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (cPTIO) were purchased from Cayman (USA).

Nitric oxide measurement

Nitric oxide concentrations were verified using enhanced chemiluminescence with a Sievers Model 280 Nitric Oxide Analyzer (Boulder, CO). Authentication of •NO as the species responsible for the observed signal was accomplished using cPTIO while positive controls were conducted with PROLI NONOate.

XOR activity

Crystallized xanthine oxidase was further purified to remove ammonium sulfate using G25 Sephadex columns (GE Health Sciences, USA) and enzymatic activity determined by the rate of uric acid formation monitored (λ = 292 nm) in potassium phosphate buffer (KPi) pH = 7.4. Units of activity are defined as: 1 Unit = 1 μmole uric acid/min.

XOR binding to heparin-Sepharose 6B (HS6B)

Purified XO was bound to HS6B as we previously described [14]. HS6B-XO was used by adding 100 μL of XO (75 mUnits/mL in pH 7.4) to the purging vessel of the Nitric Oxide Analyzer containing 5 mL of KPi pH 6.5. Thus, the final working concentration of HS6B-XO activity was 1.5 mUnits/mL.

Aldehyde oxidase

Incubations were performed using a technique previously described by Barr and Jones [15]. Briefly, incubation mixtures consisted of N-[2-(dimethylamino)ethyl]acridine-4-carboxamide (DACA, 6 μM in DMSO), febuxostat (50–1000 μM in DMSO), 25 mM potassium phosphate buffer with 0.1 mM EDTA (pH 7.4) in a final reaction volume of 800 μL. Reactions were initiated by addition of human liver cytosol (HLC) to achieve a final concentration of 0.05 mg protein/mL. The final DMSO concentration in assay was 1% (v/v), which has no effect on the reaction [16]. Reactions were allowed to proceed for 5 min at 37 °C and subsequently quenched with 200 μL of 1.0 M formic acid containing a known concentration of 2-methyl-4(^{3}H)-quinazolinone as internal standard. Quenched samples

were centrifuged at 5000 rpm for 10 min in a 5415D Eppendorf centrifuge and the supernatant collected for analysis by LC–MS/MS [15].

Statistics

Data were analyzed using one way analysis of variance followed by Tukey's range test for multiple pair-wise comparisons. Significance was determined as $p < 0.05$.

Results

Purified xanthine oxidase was exposed to various concentrations of raloxifene (0–100 μM) in the presence of xanthine (100 μM) and monitored for uric acid formation, Fig. 1A. Raloxifene inhibited XO-catalyzed xanthine oxidation to uric acid in a concentration-dependent manner achieving complete inhibition near 100 μM. Inhibition of XO with allopurinol is also shown for comparison. Plotting the inverse of initial reaction velocity ($1/V_0$) versus the concentration of inhibitor (Dixon Plot) revealed a competitive inhibition process with a K_i = 13 μM for raloxifene, Fig. 1B. Examination of the effects of pH (5.5–9) on inhibition strength demonstrated greater potency for raloxifene at lower pH; values similar to those encountered *in vivo* under hypoxia/inflammation, Fig. 1C. The time to inhibition was found to be rapid with no observable difference between 0 and 60 s, Fig 1D.

To assess the capacity of raloxifene to inhibit XO-catalyzed NO_2^- reduction to $^{\bullet}NO$, purified XO was bound to heparin-Sepharose 6B beads (HS6B-XO) and added to the reaction chamber of the Nitric Oxide Analyzer containing 1 mM NO_2^- and 20 μM xanthine as depicted in Fig. 2A. Immobilization of XO on artificial glycosaminoglycans (GAGs) such as HS6B facilitates NO_2^- reductase activity and serves to protect the enzyme from degradation induced by the physical action of the flow-through purging process. After attainment of a rate of $^{\bullet}NO$ formation, the inhibitor was added and measurements were taken. Results for raloxifene, menadione, and the XO-specific inhibitor febuxostat are plotted as percent inhibition versus inhibitor concentration, Fig 2B. These data produced EC_{50} values as follows: raloxifene (64 μM), menadione (60 nM), and febuxostat (4 nM). Experiments using 100–500 μM NO_2^- produced similar results but with greater variability and diminished window of opportunity for observing signal diminution by inhibition (not shown). Experiments whereby HS6B-XO was exposed to the inhibitor before reaction initiation produced similar results (not shown). Control experiments where either the inhibitor or DMSO (solvent for inhibitors) was exposed to decaying PROLI NONOate produced no observable diminution of signal indicating the absence of direct actions between inhibitor/solvent and $^{\bullet}NO$.

To examine potential inhibitory actions of febuxostat for AO, human liver cytosol was exposed to various concentrations of febuxostat and assessed for using 6 μM of the AO selective substrate N-[2-(dimethylamino)ethyl]acridone-4-carboxamide (DACA) [15], Fig. 3. Plotting percent inhibition versus febuxostat concentration revealed an IC_{50} of 613 μM with complete inhibition occurring at levels over 1 mM.

Discussion

The potential therapeutic impact of NO_2^- – mediated enhancement of •NO bioavailability is evolving rapidly as reports of salutary actions of NO_2^- treatment are appearing at steady rate. As such, understanding the reductive processes driving this alternative •NO pathway is vital. The molybdopterin-containing enzymes XO and AO have been identified as potential contributors to this pathway by demonstrating NO_2^- reductase activity under conditions similar to those that diminish the •NO production capacity of nitric oxide synthase; hypoxia and acidic pH. However, as stated above, several factors coalesce to provide significant obstacles to successfully assigning relative contributions to •NO formation to AO and XO in cell and tissue systems affirming the need for a more viable approach.

Previous reports have indicated potent inhibition (K_i = 1.0–51 nM, depending on the reducing substrate) properties of raloxifene for AO and thus this compound has been used to explore AO-mediated biochemistry including NO_2^- reduction [4,13,16]. However, there exists no detailed analysis regarding crossover inhibition of XO by raloxifene. Herein, we tested raloxifene for capacity to inhibit XO-catalyzed xanthine oxidation to uric acid and found significant inhibition (K_i = 13 μM) suggesting that application of raloxifene to specifically inhibit AO at concentrations near this level would induce considerable inhibition of XO. In addition, inhibition of XO by raloxifene was more pronounced under slightly acidic conditions similar those encountered in a hypoxic/inflammatory milieu. More importantly, it was determined that raloxifene inhibits XO-catalyzed NO_2^- reduction with albeit less potency (EC_{50} = 64 μM) than that observed for xanthine oxidation to uric acid. However, inhibition of XO-dependent NO_2^- reduction was not observed below 1.0 μM suggesting that application of raloxifene at concentrations up to 1.0 μM would serve to completely inhibit AO while not altering XO-catalyzed reactions. It is important to note that menadione, a commonly used alternative to raloxifene for AO inhibition analysis, did not alter XO-mediated uric acid oxidation; yet, it did potently inhibit XO-catalyzed NO_2^- reduction to •NO (EC_{50} = 60 nM) [17,18]. It is also crucial to note that we are not endorsing the use of raloxifene for *in vivo* studies as it is an estrogen receptor antagonist and thus not an AO-specific inhibitor.

Combined, these data suggest that application of raloxifene at sub-•M concentrations is an appropriate strategy for discerning AO-catalyzed NO_2^- reduction from that mediated by XOR in cell culture and *ex vivo* tissue experimentation whereas the use of menadione should be avoided.

Febuxostat (Uloric®) has been identified as an XOR-specific inhibitor that: (1) is 3 orders of magnitude more potent than the classical pyrazalopyrimidine-based XO inhibitor allopurinol (K_i = 0.96 nM vs. 0.7 μM) and (2) unlike allo/oxypurinol, is not affected by XO-endothelial GAG interactions and does not affect alternative purine catabolic pathways [12,19]. However, there have been no reports investigating potential inhibitory action of febuxostat on AO. Herein, we report febuxostat to be a superior inhibitor of XO-catalyzed NO_2^- reduction (EC_{50} = 4 nM) while demonstrating very poor inhibition properties for AO (EC_{50} = 613 μM). In addition, our previous studies revealed no interaction between DACA and XO

affirming no interference of XO catalyzed reactions and DACA catabolism [20]. These data suggest that application of febuxostat to specifically inhibit XO-catalyzed NO_2^- reduction would be an appropriate approach as febuxostat is not only superior to allopurinol but does not alter AO Mo-co-catalyzed reactions.

In toto, limitations including the absence of genetic knockout models have relegated investigators to employ pharmacologic means to distinguish between XOR- and AO-catalyzed reactions. Of developing importance is the capacity to distinguish between XOR- and AO-catalyzed reduction of NO_2^- to $^{\bullet}NO$ in cell culture and tissues. Herein, we report that sub-μM concentrations of raloxifene will serve to specifically inhibit AO while concentrations of febuxostat below 100 μM will specifically inhibit XOR in the absence of either inhibitor participating in observable crossover inhibition.

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Abbreviations

AO	aldehyde oxidase
GAGs	glycosaminoglycans
H_2O_2	hydrogen peroxide
$^{\bullet}NO$	nitric oxide
NOS	nitric oxide synthase
$O_2^{\bullet-}$	superoxide
RNS	reactive nitrogen species
ROS	reactive oxygen species
XDH	xanthine dehydrogenase
XO	xanthine oxidase
XOR	xanthine oxidoreductase

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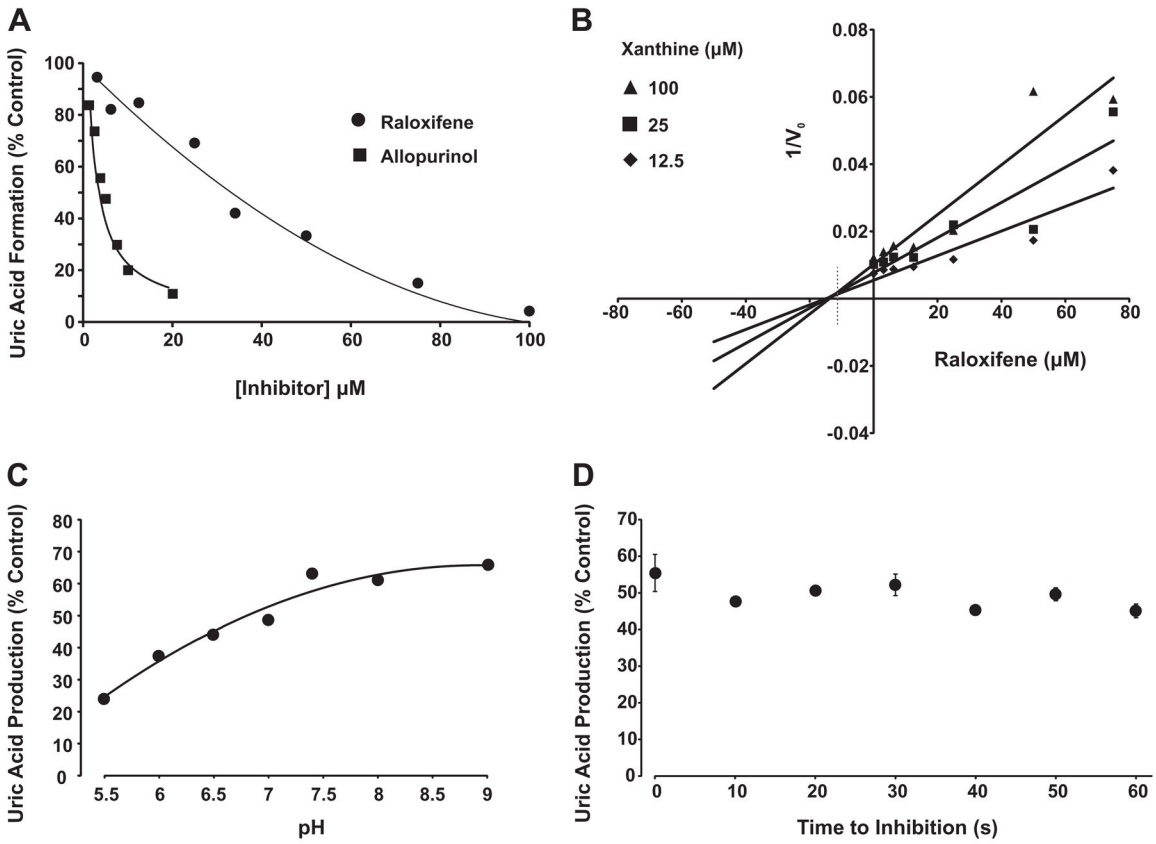

Fig. 1.

Raloxifene inhibition of XO. (A) Purified xanthine oxidase (2 mUnits/ml, phosphate buffered saline, pH 7.4) was exposed to various concentrations of either raloxifene or allopurinol and assessed for formation uric acid (λ = 295 nm) upon the addition of xanthine (100 μM). Shown are the initial reaction rates (V_0) plotted as % control (no inhibitor). (B) Purified xanthine oxidase (as above) was exposed to various concentrations of raloxifene and assessed for formation uric acid (λ = 295) using 3 concentrations of xanthine (12.5, 25, and 100 μM). Shown is a Dixon plot ($1/V_0$ vs. [inhibitor]) generating an inhibition constant (K_i) = 13 μM for raloxifene and demonstrating a competitive inhibition process defined as line intersection above the abscissa (*dashed line*). (C) Purified xanthine oxidase (as above) was exposed to a single concentration of raloxifene (37 μM) and assessed for the effect of pH (5.5–9.0) on inhibition capacity. (D) Purified xanthine oxidase (as above) was exposed to a single concentration of raloxifene (37 μM) and assessed for time to inhibition (0–60 s). For (A–C), data represent the mean of at least 4 independent determinations. For (D), data represent the mean and standard deviation of at least 3 independent determinations with no significant difference observed at 95% confidence, $p > 0.05$.

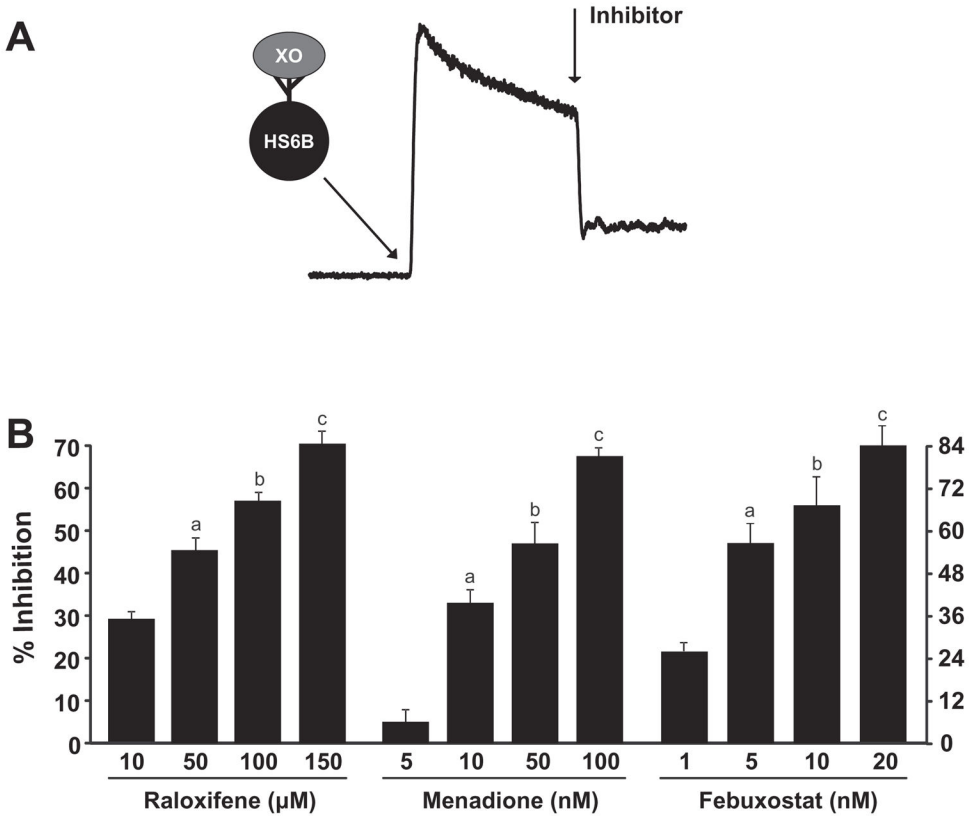

Fig. 2.

Inhibition of XO-catalyzed NO_2^- reduction to •NO. (A) Diagram depicting a typical experiment where HS6B-XO (final concentration = 1.5 mUnits/mL) was added to the reaction vessel of the Nitric Oxide Analyzer containing 1 mM $NaNO_2^-$ and 20 μM xanthine. After establishment of a rate of •NO generation, the inhibitor was added. All inhibitors were added at the same time after the addition of HS6B-XO (B) Inhibition profiles for raloxifene, menadione, and febuxostat generating EC_{50} values of 64 μM, 60 nM and 4 nM, respectively. For raloxifene a–c are significantly different from 10 μM as well as between groups $p < 0.05$. For menadione a–c are significantly different from 5 nM as well as between groups $p < 0.05$. For febuxostat, a–c are significantly different from 1.0 nM, $p < 0.05$ and a is significantly different from c, $p < 0.05$.

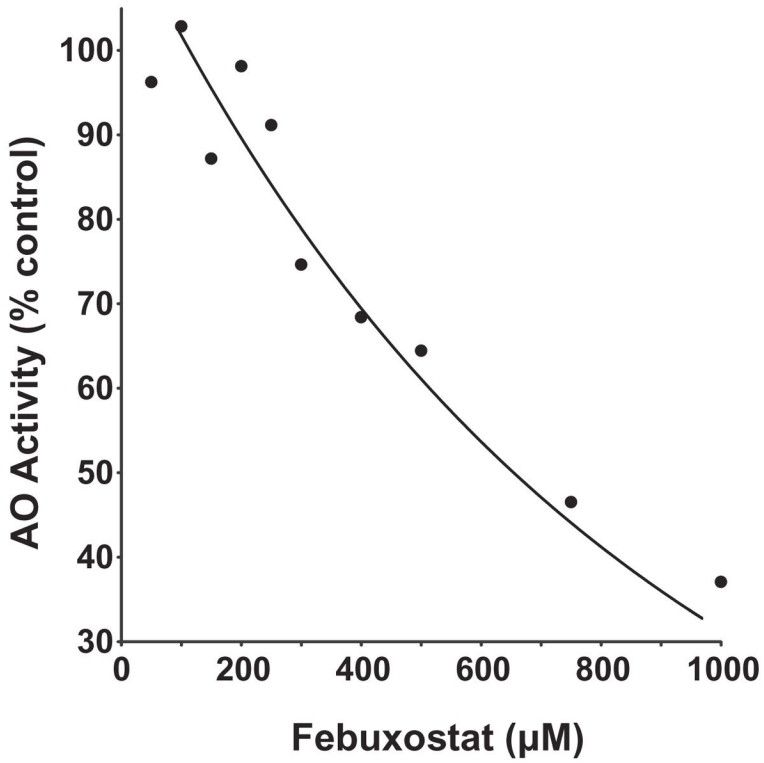

Fig. 3.

Febuxostat inhibition of AO. The fractional inhibition of the AO-specific substrate DACA in human liver cytosol (0.05 mg protein/ml) exposed to various concentrations of febuxostat (0–1 mM). The DACA concentration was fixed at 6 μM and reaction progress determined by LC–MS/MS. The IC_{50} for febuxostat inhibition of DACA metabolism was determined to be 613 μM. Data represent the mean of at least 3 independent determinations.