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Identification of potent inhibitors of the chicken soluble epoxide hydrolase

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ABSTRACT

In vertebrates, soluble epoxide hydrolase (sEH) hydrolyzes natural epoxy-fatty acids (EpFAs), which are chemical mediators modulating inflammation, pain, and angiogenesis. Chick embryos are used to study angiogenesis, particularly its role in cardiovascular biology and pathology. To find potent and bio-stable inhibitors of the chicken sEH (chxEH) a library of human sEH inhibitors was screened. Derivatives of 1(adamantan-1-yl)-3-(*trans*-4-phenoxycyclohexyl) urea were found to be very potent tight binding inhibitors (K_i <150 pM) of chxEH while being relatively stable in chicken liver microsomes, suggesting their usefulness to study the role of EpFAs in chickens.

In mammals, epoxides of arachidonic acids (called epoxy-eicosatrienoic acids or EETs) and of other fatty acids are important lipid mediators that have key roles in the regulation of hypertension, inflammation, and many cardiovascular related diseases as well as in modulating both inflammatory and neuropathic pain.[1,2] However, endogenous metabolism of these epoxy-fatty acids to their corresponding hydrated products by soluble epoxide hydrolase (sEH EC 3.3.2.10) generally reduces these biological activities.[3–5] Both in vitro and in vivo studies have demonstrated that the anti-hypertensive and cardio protective effects mediated by the EETs are inversely dependent on the extent of their hydrolysis by sEH.3-5 Thus, maintaining a high in vivo concentration of EETs through sEH inhibition is a promising therapeutic pathway to treat cardiovascular and other diseases.[3–5] Recently, EETs have been shown to promote angiogenesis in humans,[2,6] while epoxides of DHA have been shown to reduce angiogenesis,[7] underlying the importance of epoxy-fatty acids in the regulation of this biological process and the potential of human sEH inhibitors to treat diseases associated with angiogenesis.

These beneficial therapeutic effects of epoxy-fatty acids have shown a potential for veterinary applications.[8] Currently, the only classes of drugs used to reduce pain and inflammation in animals are opioids and nonsteroidal anti-inflammatory drugs (NSAIDs). Testing sEHIs for veterinary purposes could increase the limited veterinary drug armamentarium. There has already been some success using sEHIs as an analgesic and anti-inflammatory for horses with laminitis.[9] Testing potential human drugs and therapies on animals is an effective way to increase the variety of available veterinary pharmaceuticals and can also give researchers insight into the potential effects of these drugs on humans.

Animal models are effective tools for the study of diseases but the high cost associated with mammalian models makes their use impractical in initial studies. Therefore, utilizing non-mammalian animal models can provide a cost effective way to study human diseases.[10] The chicken and chick embryo model has been used in research since the time of Aristotle.[11] More recently, chickens have been successfully used as a model for various human diseases.[11] Beside being classically used for immunology, genetics, virology, cancer, and cell biology, chick embryos are currently also being used as a human model for angiogenesis and its role in cardiovascular biology and pathology.[12] Interestingly, a dose dependent vascular response to EETs was observed in chickens.[13] In addition, chicken sEH (chxEH) is active on EETs and the catalytic residues between chxEH and human sEH are conserved.[14] The overall selectivity of chxEH for a series of epoxy-fatty acids (Fig. 1) is similar to the human sEH,[15] with a clear preference for the epoxide of DHA over the epoxides of EPA, ARA or linoleic acid. The kinetic constants for chxEH's best substrate, 16,17-epoxy-docosapentaenoic acid, yield a K_m (12 ± 3 μM) that is similar to the one of the human sEH, but a V_{max} (728 ± 97 nmol min^{-1} - mg^{-1}) that is roughly 10-fold lower than the one measured for

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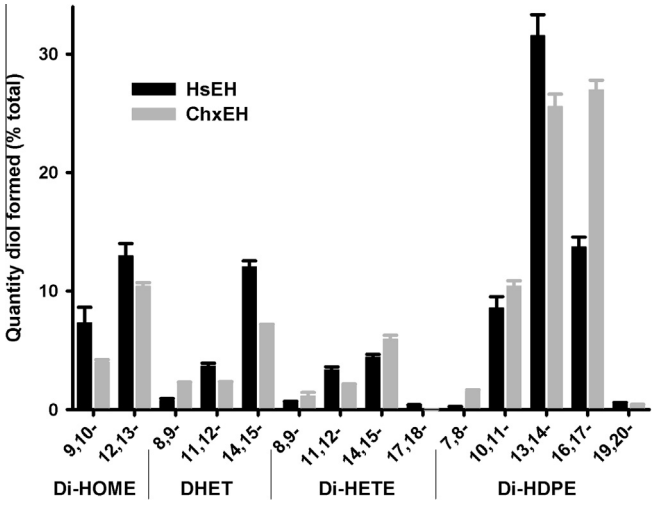

Figure 1. Substrate preferences of human and chicken sEH. Selectivity was measured using a mixture of 14 epoxy-fatty acids each at a concentration of 1 μM, and the diols formed were quantified by LC/MS–MS.[15] Di-HOME: diols from linoleic acid epoxides; DHET: diol from arachidonic acid epoxides; Di-HETE: diols from EPA epoxides; Di-HPDE: diols from DHA epoxides.

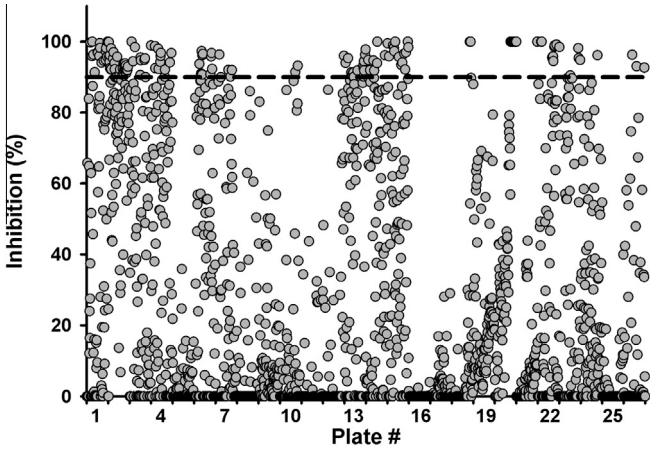

Figure 2. Primary screening results of the EH inhibitor library. Percent of sEH inhibition for each compound tested at 100 nM. Compounds that gave more than 90% inhibition (dashed line) were selected for secondary screening.

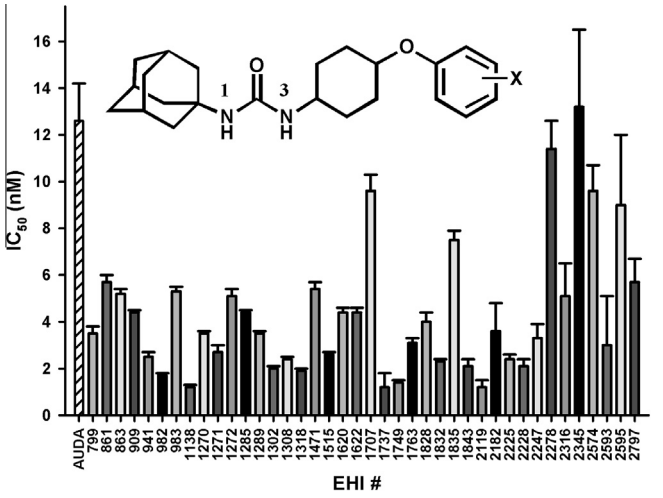

Figure 3. Secondary screening results. IC_{50}s were measured using PHOME as substrate.[18] Results are mean ± standard deviation of at least three separate measurements. The general structure of the most common inhibitor is given.

the human sEH.[16] Finally, epoxy-fatty acids were detected in the plasma and liver extracts of chicken.[14] Put together, these data support using the chick embryo model to study the role of epoxy-fatty acids in cardiovascular angiogenesis, especially to quickly and cheaply test the pharmacological action of sEH inhibitors.

A small series of sEH inhibitors were previously tested on chxEH,[14] however the more potent inhibitors found are either metabolically unstable in vivo or have low solubility limiting their usefulness, though as compounds become more potent, solubility is less important, of course. Thus, toward finding more potent and more useful chxEH inhibitors, we herein report the screening of a chemical library of EH inhibitors.[17] This library is a unique collection of over 2,200 chemicals (26 plates of 88 compounds at 10 mM in DMSO) that were synthesized with the aim of inhibiting mammalian soluble epoxide hydrolases.

Using recombinant purified chicken sEH and the fluorescent substrate PHOME ((3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester; $K_m = 1.5 \pm 0.3$ μM, and $V_{max} = 60 \pm 4$ nmol min^{-1} mg^{-1}), we screened the library at a final concentration of inhibitor at 100 nM and a chxEH concentration of 1.4 nM (84 ng/mL), following a methodology previously described for the human sEH.[18,19] Overall, we obtained on average for the 26 plates S/B = 2.9 ± 0.3, S/N = 100 ± 60 and $Z' = 0.81 \pm 0.07$ indicating that the assay performed very well.

As shown in Figure 2, out of the 2288 compounds that composed the EH inhibitor library, 200 showed greater than 90% inhibition for chxEH. To confirm the potency of these compounds, fresh solutions in DMSO were prepared, and their ability to inhibit the chicken sEH was tested at 100, 10 and 1 nM using PHOME as substrate. Instead of the endpoint mode used for the primary screening, a kinetic mode was used to eliminate compounds that gave false positive responses by altering the fluorescent signal.[19] Out of 196 compounds tested, 99 gave high inhibition (>75%) at 100 nM, suggesting a rate of false positive around 50% in the primary screen. Of these, around 40 yielded more than 50% inhibition at 10 nM, which is roughly the inhibitory potency (IC_{50}) of AUDA, the best chxEH previously described.[14] The IC_{50}s of these 40 compounds were determined using PHOME as substrate for chxEH (Fig. 3, structures are given in Table S1).[19]

The IC_{50}s of these 40 compounds for chxEH were compared with the ones obtained previously for the human sEH. A Spearman's ranking correlation coefficient (ρ) of only 0.17 was found, suggesting significant differences in the actives sites of the two enzymes. As shown in Figure 3, most of the compounds tested yielded IC_{50}s significantly smaller than AUDA's 12 nM, indicating that we successfully identified more potent chxEH inhibitors. When looking at the structure of the 40 compounds, general structural features necessary to yield potent chxEH inhibitors emerged. The library contains mostly ureas (1602 out of 2288) and amides (450 out of 2288). However, mostly urea compounds (37 out of 40) were obtained as potent chxEH inhibitors, suggesting that, as observed for the human sEH,[20] a central urea pharmacophore is needed to yield more potent chxEH inhibitors. The other 3 compounds are amides, suggesting this functionality as an alternative central pharmacophore for chxEH inhibitors. On the 1-position (the smaller side) of the urea function, the compounds in the library have mostly aryl (954 out of 1602) or adamantyl (403 out of 1602) substituents followed by cycloalkyl (144 out of 1602) and alkyl chains (101 out of 1602). Interestingly, of the potent chxEH inhibitors obtained in this screen, adamantyl functionality was the most frequent (20 out of 37 ureas), followed by aryl sub-