Published in final edited form as:
*Bioorg Med Chem Lett*. 2015 January 15; 25(2): 276–279. doi:10.1016/j.bmcl.2014.11.053.

# Identification of potent inhibitors of the chicken soluble epoxide hydrolase

**Diyala S. Shihadih**[a], **Todd R. Harris**[a], **Jun Yang**[a], **Oleg Merzlikin**[a], **Kin Sing S. Lee**[a], **Bruce D. Hammock**[a], and **Christophe Morisseau**[a,*]

[a]Department of Entomology and Nematology, and U.C. Davis Comprehensive Cancer Center, University of California Davis, Davis, CA 95616 USA

## 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.

### Keywords

epoxy-eicosatrienoic acids; angiogenesis; liver microsomes; high throughput screening; di-substituted ureas

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



*Corresponding author. Tel.: +1-530-752-6571; fax: +1-530-752-1537; chmorisseau@ucdavis.edu.

Supplementary Material
Structures of the 40 potent chxEH inhibitors obtained are given in Supplementary material, as well as the details of the mass spectrometric method used to determine microsomal stability.

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 (Figure 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 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 ± 0.3 μM, and $V_{max}$= 60 ± 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 ± 0.07 indicating that the assay performed very well.

As shown in Figure 2, out of the 2,288 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 (Figure 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 functionallity 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 folowed by cycloalkyl (144 out of 1602) and alkyl chains (101 out of 1602). Interestingly, of the potent chxEH inhibitors obtained in this screen, admantyl functionality was the most frequent (20 out of 37 ureas), followed by aryl substituents (13/37) and cycloalkyl (4/37). These suggest that, contrary to the human sEH,[20] an adamantyl group is more likely to yield potent compounds for chxEH inhibition than phenyl groups. However, exceptions exist such as compound **1749**. On the 3-position of the urea function, the library contains a similar amount of compounds with alkyl (280), phenyl (358), cyclohexyl (364) or piperidyl (280) substituents. However, for the chxEH, a cyclohexyl group was the most frequent (25/37), while alkyl, piperidyl and phenyl derivatives were less common (7/37, 2/37 and 3/37, respectively). In general, piperidine ureas are potent human sEH inhibitors.[20] Remarkably, this series of compounds are not potent against chxEH, except for **1749**, underlying difference in selectivity between the chicken and human enzymes. For example, the commonly used piperidine urea TPPU (aka 1770)[5] showed only 15% inhibition fo the chxEH at 100 nM. Interestingly, most of the inhibitors (29/37) for chxEH found (table S1) have an ether function as secondary pharmacophore independently of the nature of the group between the urea and the secondary polar function. The library

contains roughly the same amount of urea compounds with an ether (454) or a carboxylate derivatives (383) as secondary pharmacophore. Finally, for the chxEH inhibitors, on the other side of this ether, aryl groups (24/30) were the more frequent followed by alkyl groups, which is similar to the compounds in the library. The resulting general structure for the most potent chxEH inhibitors is shown in Figure 3. While the structure depicted in Figure 3 is similar to a series of inhibitors developed for the human sEH,[21] it does not depict the most potent human sEH inhibitors,[20] underlying significant differences between the selectivity of the human and chicken enzymes.

While it is important to identify novel potent inhibitors of chxEH in an *in vitro* screen, the compounds need also to be soluble and metabolically stable for *in vivo* use. These parameters were previously shown to greatly influence the *in vivo* potency of sEH inhibitors in rodents.[22] Thus, five newly identified chxEH inhibitors with distinct structural features were chosen, along with AUDA as a positive control, for further testing.

The cLogP, solubility and stability were determined for all seven compounds (Table 1). The cLogPs were calculated using ChemBioDraw (v.12.0, CambridgeSoft). The cLogP is a measurement of both relative solubility and bioavailability. For **AUDA** and **1471** (a.k.a. *t*-AUCB), the charged form (a.k.a. base form) of the acid function yields significantly lower cLogP, suggesting higher water solubility for the base form, as expected. Otherwise, compounds **1749**, **2119**, **2225** and **2228** have cLogP between 3 and 4, suggesting reasonable solubility and good bioavailability.[23] To support these results, the solubility in sodium phosphate buffer (0.1 M pH 7.4) containing 1% of DMSO was determined by turbidity measurement at 600 nm, as described.[24] Compared to **AUDA**, compounds **1471**, **1749**, **2119** and **2225** are markely more soluble, suggesting they will be easier to use. Solubility seems to be enhanced by the presence of an adamantyl (**2225** vs **2228**) and an acid function (1**471** vs **2225**). The stability of the seven inhibitors (1 μM) in both chicken liver microsomes and human liver microsomes (1 mg/mL) was measured in the presence or absence of NADPH as described.[25] After a 30 minute incubation at 37°C, the amount of remaining inhibitor was quantified using mass spectrometry.[25] Of the seven compounds tested, four of them, **AUDA**, **1471**, **2119** and **2225**, display similar stability in both human and chicken liver microsomes. Interstingly, **1749** shows a great disparity between its stability in human and chicken liver microsomes. While it very stable in the chicken microsomes, it was completely metabolized in 30 min by the human microsomes. Because this occurs in the presence and absence of the NADPH regenerating system, it suggests that **1749** metabolism in human microsomes is not P450-dependent. Because **1749** contains an ester, its rapid metabolism probably resulted from the action of esterases, such as carboxylesterases that are well known to be present in liver microsomes.[26] Our results suggest that the selectivity of these human esterases is quite different than the chicken esterases. In the presence of NADPH, **1138** was rapidly metabolized in the chicken liver microsomes (2% left after 30 min), while half of it remain after 30 min in contact with the human liver microsomes (Table 1). Similarly, with NADPH, **2228** is rapidly metabolized by the human liver microsomes, but not by the chicken liver microsomes, underlying the difference in selectivity of the human and chicken microsomal enzymes.

Put together, the combined results from Table 1 suggest that compounds **1471**, **2119** and **2225** have the best set of properties making them excellent inhibitors of chxEH for use in chick embryo or in chicken. To confirm the high inhibitory potency of these compounds, their dissociation constants ($K_I$) for chxEH were determined using a Förster resonance energy transfer (FRET)-based competitive displacement assay.[27] The reporter ligand used is this assay is 1-(adamantan-1-yl)-3-(1-(2-(7-hydroxy-2-oxo-2H-chromen-4-yl)acetyl) piperidin-4-yl) urea (ACPU). Using our recombinant purified (> 95% pure) chxEH,[14] a $K_D$ of 283 pM for ACPU was found. Using this value, the $K_I$s were calculated by non-linear regression from the displacement curve of chxEH–ACPU complex by increasing amount of the tested inhibitor (Figure 4).[27] A $K_I$ of 132 ± 1 pM was obtained for **1471**, while $K_I$s of less than 20 pM (the limit of quantification) were obtained for **2119** and **2225**, supporting the inhibtory potency of the selected compounds.

In conclusion, our results show that while the substrate selectivity for the human sEH and chxEH are similar, the enzymes' inhibitor selectivities are quite different. In addition, the microsomal metabolic stabilities of a few inhibitors were quite different between the human and chicken liver. Nevertheless, we identified three chxEH inhbitors, **1471**, **2119** and **2225**, that are very potent, have suitable physical properties, and are metabolically stable in chicken liver microsomes, making them suitable for future use in chicken models of disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported in part by NIEHS grant ES02710 and NIEHS Superfund grant P42 ES04699. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors want to thank Dr Huaijun Zhou for the gift of chicken liver.

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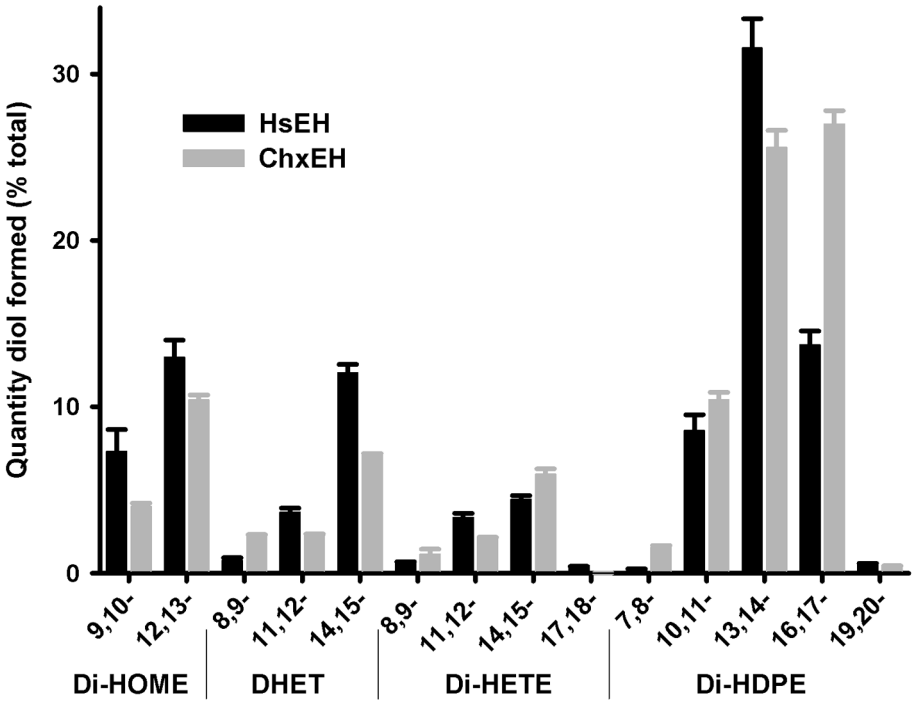



**Figure 1.**

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.

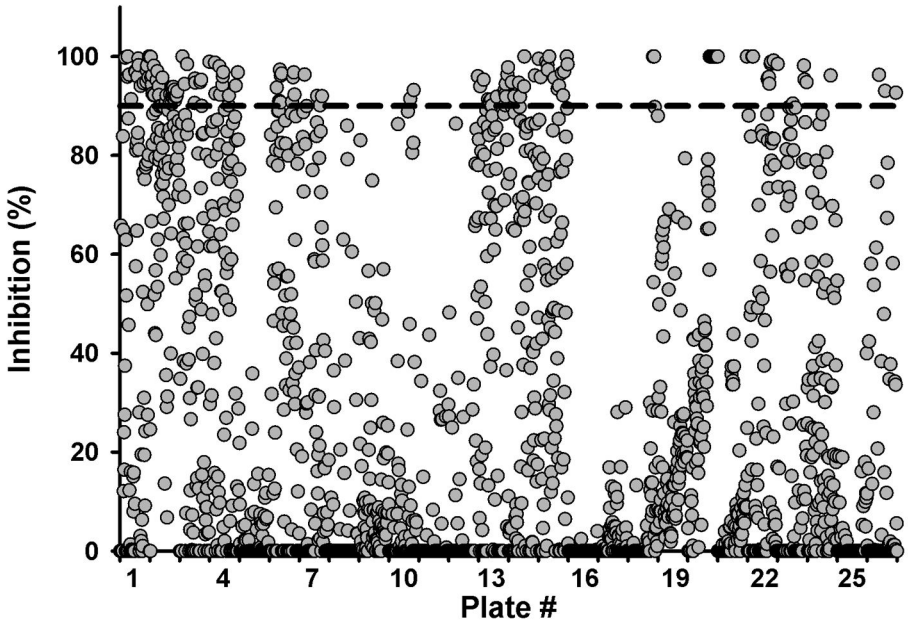



**Figure 2.**

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.

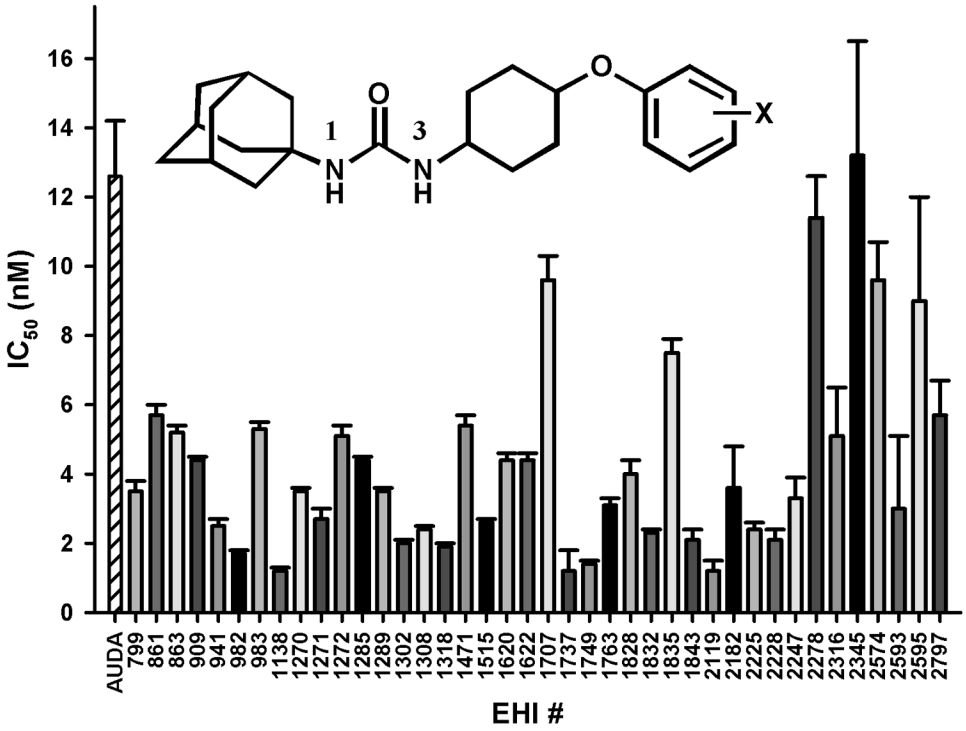



**Figure 3.**

Secondary screening results. $IC_{50}$s were measured using PHOME as substrate.[19] Results are mean ± standard deviation of at least three separate measurements. The general structure of the most common inhibitor is given.

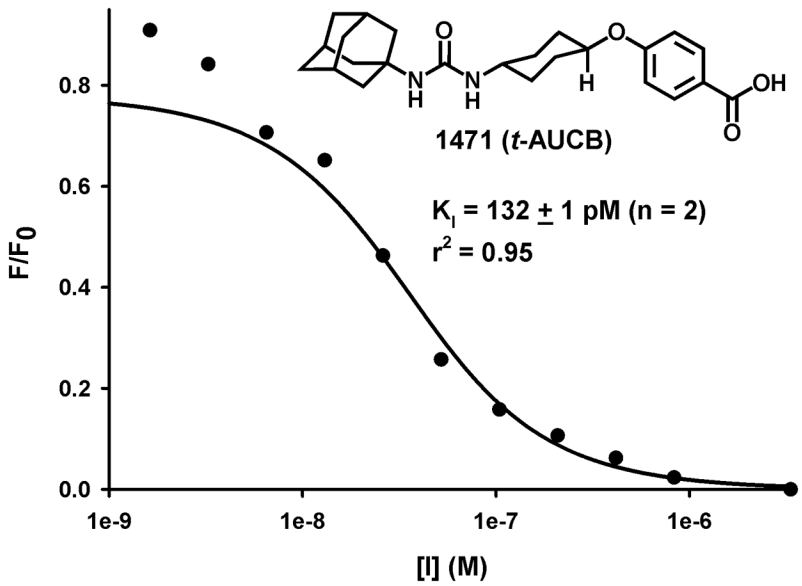
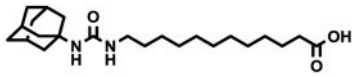
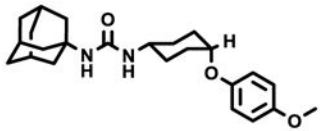
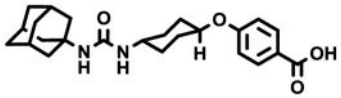
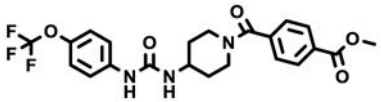
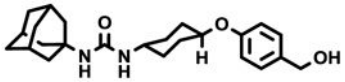
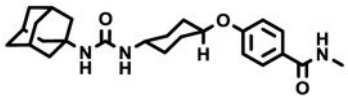
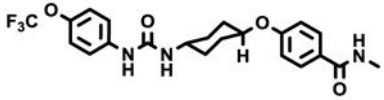



**Figure 4.**

Displacement curve of chxEH–ACPU complex with EH inhibitor #**1471**. [I] are shown in log scale.

**Table 1**

cLogP, solubility and stability of selected compounds.

| Structure | EHI# | cLogP | Solubility (μM)[b] | Stability (%)[c] | | | |
|---|---|---|---|---|---|---|---|
| | | | | Human liver mic. | | Chicken liver mic. | |
| | | | NADPH | − | + | − | + |
|  | **AUDA** | 5.98<br>−1.14[a] | 37.5–50 | 98 ± 14 | 69 ± 5 | 106 ± 7 | 70 ± 1 |
|  | **1138** | 4.97 | 10–12.5 | 98 ± 3 | 57 ± 21 | 69 ± 29 | 2.1 ± 0.5 |
|  | **1471** | 4.84<br>0.62[a] | > 100 | 97 ± 8 | 69 ± 2 | 77 ± 2 | 60 ± 4 |
|  | **1749** | 2.90 | 75–100 | 1.2 ± 0.5 | 2.7 ± 0.8 | 105 ± 9 | 94 ± 4 |
|  | **2119** | 3.84 | > 100 | 91 ± 6 | 53 ± 2 | 73 ± 1 | 24 ± 2 |
|  | **2225** | 3.88 | 50–75 | 101 ± 4 | 94 ± 2 | 96 ± 5 | 96 ± 3 |
|  | **2228** | 4.08 | 10–12.5 | 101 ± 5 | 5.4 ± 0.1 | 119 ± 15 | 89 ± 15 |

[a]Base form.

[b] In sodium phosphate buffer (0.1 M pH 7.4)

[c] Percent of compound (1 μM) remaining after 30 minutes incubation with human or chicken liver microsomes (1 mg/mL) at 37°C with or without NADPH generating system. Results are average ± standard deviation (n = 3).