

## Review

# Gerry Brooks and epoxide hydrolases: four decades to a pharmaceutical

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**Abstract:** The pioneering work of Gerry Brooks on cyclodiene insecticides led to the discovery of a class of enzymes known as epoxide hydrolases. The results from four decades of work confirm Brooks' first observations that the microsomal epoxide hydrolase is important in foreign compound metabolism. Brooks and associates went on to be the first to carry out a systematic study of the inhibition of this enzyme. A second role for this enzyme family was in the degradation of insect juvenile hormone (JH). JH epoxide hydrolases have now been cloned and expressed from several species, and there is interest in developing inhibitors for them. Interestingly, the distantly related mammalian soluble epoxide hydrolase has emerged as a promising pharmacological target for treating hypertension, inflammatory disease and pain. Tight-binding transition-state inhibitors were developed with good ADME (absorption, distribution, metabolism and excretion). These compounds stabilize endogenous epoxides of fatty acids, including arachidonic acid, which have profound therapeutic effects. Now EHs from microorganisms and plants are used in green chemistry. From his seminal work, Dr Brooks opened the field of epoxide hydrolase research in many directions including xenobiotic metabolism, insect physiology and human health, as well as asymmetric organic synthesis.

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**Keywords:** xenobiotic metabolism; juvenile hormone; cardiovascular diseases; asymmetric organic synthesis

## 1 INTRODUCTION TO, AND HISTORY OF, EPOXIDE HYDRATION

This article is somewhat outside the usual scope of *Pest Management Science* in that it traces aspects of the scientific career of our long-term editor, Gerry Brooks. In particular, this paper focuses on his pioneering work on epoxide hydrolases (EHs) and briefly describes how these early studies have evolved into multiple fields including xenobiotic metabolism, drug–drug interaction, pesticide chemistry, herbicide chemistry, asymmetric organic chemistry, insect developmental biology and even a new pharmaceutical target to address pain, inflammation and hypertension. Gerry has never restricted himself to a single species, and subsequent work on epoxide hydrolase reflects this tradition, with increasing numbers of articles on this enzyme in birds, plants, nematodes, fungi, bacteria and, of course, mammals and insects. Certainly, this is a clear example of a field, of which Gerry was a founder, that has expanded in numerous and often unexpected directions.

Like many scientists, Gerry has had a varied career, starting as a steroid chemist and making major contributions in areas as diverse as insect endocrinology and pesticide resistance. Possibly Gerry is best known for his comprehensive work on chlorinated pesticides.<sup>1,2</sup> In particular, Gerry worked with the cyclodiene insecticides. These compounds

had a major positive impact on food production and human health. However, the high persistence and toxicity of some members of this class of compounds led to concerns over the cyclodienes. This concern was a major driver of the environmental movement leading to the establishment of the US Environmental Protection Agency (EPA) and the current regulatory systems worldwide on pesticide usage. As an interesting aside, the metabolism of organochlorine pesticides described by Brooks in the early 1960s and summarized in his 1974 book<sup>2</sup> define unique target sites for pest control. Over half a century later, they not only remain viable targets but in fact are the site of action of a variety of insect control agents that are valuable in integrated pest management and have a high degree of both human and environmental safety. These targets are discussed elsewhere in this issue. This paper focuses on an aspect of Gerry's career that branched off from his work on cyclodiene insecticides. Many of the major cyclodienes were found to be epoxidized, and the resulting oxiranes turned out to be surprisingly stable, often very active biologically and in some cases insecticides in their own right, such as dieldrin. Gerry and others found that the cyclodienes were in fact metabolized by microsomal hydroxylation, epoxidation and in a few cases by epoxide hydration.<sup>2</sup> This observation on enzymatic hydration of cyclodiene epoxides led to a series of

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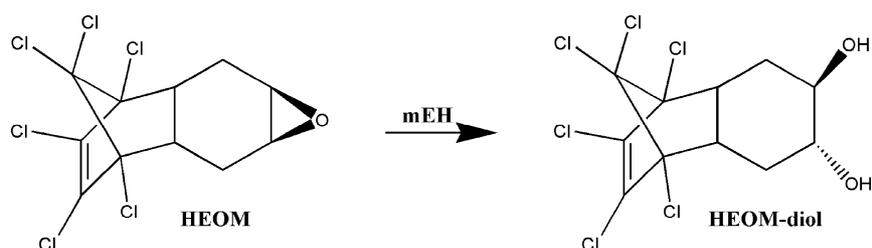
seminal papers in the epoxide hydrolase field. These papers resulted in the recognition of the microsomal epoxide hydrolase as a key player in xenobiotic metabolism in mammals and insects. They also led to some pioneering work in insect endocrinology and a general appreciation for epoxide hydration.

The first comprehensive article on the enzyme epoxide hydrolase (E.C.3.3.2.3, formerly epoxide hydratase or epoxide hydrase) was published by Brooks in 1970.<sup>3</sup> Prior to this, Williams<sup>4</sup> and Boyland<sup>5</sup> suggested independently the existence of an enzyme that added water to epoxides from their studies of the metabolism of aromatic compounds. By the late 1960s, several laboratories working in other areas had established the involvement of an epoxide hydrolase in epoxide ring opening with compounds that they were studying.<sup>6,7</sup> The manuscript by Brooks *et al.*<sup>3</sup> in 1970 was a landmark study for many reasons. At the time of its publication, cyclodienes were major pest control agents, and the demonstration of diol products was important for understanding their fate. More generally, the great stability of cyclodiene epoxides firmly established epoxides as key intermediates in the olefin-to-diol conversion, as had been suggested for less stable epoxides.<sup>6-9</sup> In their study, Brooks *et al.*<sup>3</sup> clearly showed the identity of the product diols by their relatively large-scale purification and characterization and the enzymatic nature of the process. This was in part shown by the optical activity of the product diols. They also demonstrated that the product diols were products of *trans* hydration and chemically identical to the corresponding synthetic diol. Although the stereochemistry of hydration did not absolutely define the catalytic mechanism, it eliminated many possible mechanisms resulting in *cis* diols. An interesting aside was Brooks' finding that squalene oxide was a poor substrate for epoxide hydration by microsomes, and noting its similarity in structure to the insect juvenile hormones. The Brooks research group presented the most complete characterization of the microsomal epoxide hydrolase (mEH) up to that time from several species from insect to mammal. Finally, Brooks demonstrated the unhindered cyclodiene HEOM as an excellent and selective substrate for mEH (Fig. 1). The high chemical stability of this epoxide and the ease of its analysis by GLC make it still an attractive surrogate substrate for mEH. The pioneering studies in that paper<sup>3</sup> certainly have stood the test of time.

From a practical standpoint, the discovery that the HEOM epoxide was quite stable in buffer but was a good substrate for epoxide hydrolase resulted in a series of papers by Brooks and colleagues not only characterizing the microsomal epoxide hydrolase but also initiating studies on inhibition of the enzyme and moving into the metabolism of insect juvenile hormone.<sup>10-14</sup> It is unlikely that industry will revisit the cyclodienes, but the insect toxicity of the asymmetric analogue of HEOM (known as HCE) suggested the possibility of producing a biodegradable yet stable and potent insecticide in this class. Moreover, if HEOM, a close non-toxic analogue of dieldrin, could be synergized by inhibiting its epoxide ring hydration, or applied to an insect lacking EH, it might become a potent toxicant. These results provided the first *in vivo* demonstration of a biological role for the enzyme, demonstrated EH inhibitors as pharmacological probes and represented the pioneering work on the generation of inhibitors of EHs that have resulted in possible human therapeutics. During this same time period, Watabe and coworkers solubilized the mEH,<sup>15</sup> as a first step towards purification,<sup>16</sup> characterized the enzyme using a variety of olefin oxides including *cis*- and *trans*-stilbene oxide<sup>17</sup> and even reported the existence of the still poorly characterized cholesterol epoxide hydrolase.<sup>18</sup> The stilbene oxides are also excellent substrates for glutathione *S*-transferase, and constitute a pair of substrates that distinguish the microsomal (*cis*) and soluble (*trans*) epoxide hydrolase from several species.<sup>19</sup> However, during this period, the interest in carcinogenesis among mammalian toxicologists led to many workers focusing on the epoxides of aromatic compounds and possible surrogates of these unstable epoxides such as styrene oxide. This may have resulted in a failure to recognize fully the major contributions made by Brooks and his coworkers on the basic chemistry and enzymology of the mammalian microsomal epoxide hydrolase.

## 2 INSECT EPOXIDE HYDROLASES

The impact of Gerry Brooks on the entire epoxide hydrolase field is impressive, but his contributions in the early days of the investigation of epoxide hydration in insects are absolutely dominant.



**Figure 1.** HEOM hydration by mEH.<sup>3</sup> HEOM is a non-commercial cyclodiene insecticide that is an excellent substrate for the mEH owing to the lack of steric hindrance of the epoxide.

## 2.1 Insect microsomal epoxide hydrolases and xenobiotic metabolism

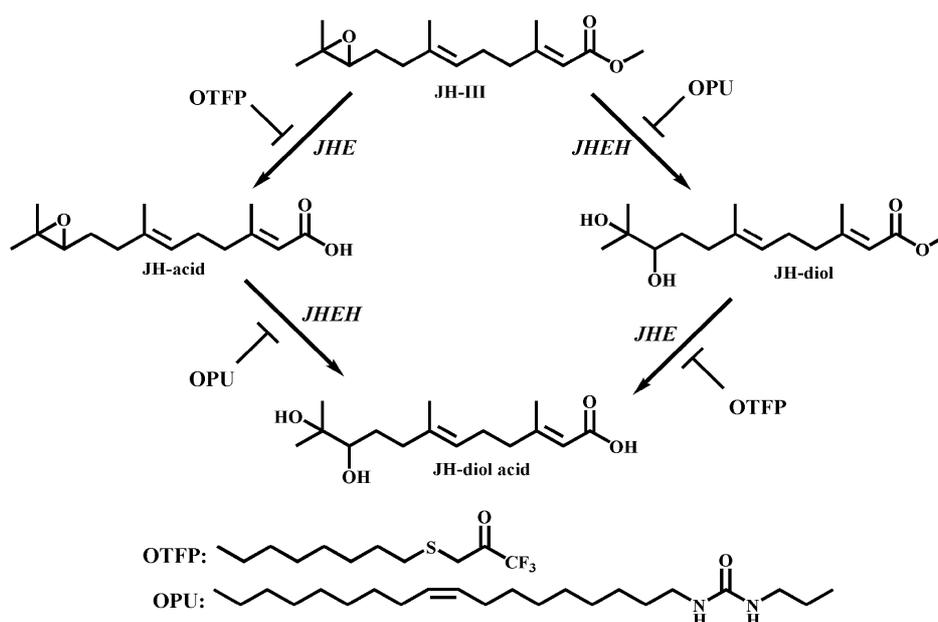
The extensive work of Gerry Brooks on both mammalian microsomal epoxide hydrolase and the epoxide hydrolases involved in JH metabolism started with an effort to find if these enzymes were involved in the degradation of epoxide-containing cyclodiene insecticides. In particular, Brooks showed an early interest in the mechanisms of resistance of insects to cyclodiene insecticides.<sup>20</sup> As discussed above, it had been found earlier that some cyclodienes can be epoxidized to yield materials that show high insecticidal activity. In fact, these epoxides represented major insecticides for many years. The work of Brooks demonstrated that these materials were resistant to epoxide hydrolases from many species, which was probably due in large part to steric hindrance blocking an attack by an enzyme nucleophile.<sup>1,2</sup> The hydration of the dieldrin and high-melting (+)-heptachlor epoxide (m.p. 160 °C) was very slow with mammalian EH but faster with the less hindered epimeric (–)-heptachlor epoxide (m.p. 89 °C).<sup>3</sup> The stability of these epoxides would later result in a disaster for the dairy industry in Hawaii.<sup>21</sup> However, a major driver for the work was to find if insect epoxide hydrolases could result in resistance to these valuable compounds. To do this, it was critical that Brooks and associates characterize the insect EHs. To date there has been no such extensive characterization of these enzymes in insects, following the work of Brooks.<sup>3</sup> A major discovery was that cyclodiene structures, which did not hinder a backside attack on the epoxide carbons, were excellent substrates not only for insect EHs but also for mammalian EHs. Additionally, because of their ease of synthesis, high hydrolytic and chemical stability, ease of analysis by GLC and excellent kinetic properties, cyclodienes (HEOM in particular) (Fig. 1)

became excellent surrogate substrates.<sup>14</sup> Of particular importance, Brooks and associates were successful in demonstrating that inhibitors of insect EHs could synergize the toxicity of the unhindered HEOM but not of hindered cyclodiene epoxides. They clearly were the first to demonstrate that epoxide hydration of xenobiotics could modulate their toxicity.<sup>11,12</sup>

Since the early work of Brooks and associates, there has been remarkably little research on insect EHs other than the JHEHs. This is in part due to there being no major commercial insecticides containing an epoxide moiety. Mullin<sup>22,23</sup> developed a nice hypothesis that epoxide hydrolases in insects may be associated with the epoxide functionality being common among plant natural products that are found in their diet. The epoxide hydrolases of *Drosophila melanogaster* (Meigen) and other flies have been investigated enough to provide a basis for future work.<sup>24–27</sup> Possibly, such model species can help to address the endogenous roles of the microsomal enzymes as well as their role in xenobiotic metabolism. It does seem sad that so very little work has been done in the area subsequent to the pioneering work of Brooks.

## 2.2 Epoxide hydrolases in the metabolism of insect juvenile hormone

Insect juvenile hormone regulates the maturation of insects, and in the hemi- and holometabola it regulates metamorphosis. It also controls a number of other events in different species, including reproduction, behaviour, coloration, diapause and other biologies. Juvenile hormone (JH) is a terpene with a conjugated methyl ester at carbon 1 and an epoxide at C10–11 (Fig. 2). At the beginning of the 1970s, Slade and Zibbit<sup>28,29</sup> first demonstrated that the major routes of metabolism of the juvenile hormones in several species



**Figure 2.** Insect JH-III metabolism by  $\alpha/\beta$ -hydrolase fold enzymes, and examples of inhibitors of these enzymes. Esterases and epoxide hydrolases in the  $\alpha/\beta$ -hydrolase fold family that act on JH are referred to as JHEs and JHEHs.

of insects were by epoxide hydration, ester cleavage or both. Both of these functionalities are relatively stable to chemical hydrolysis, and the enzymes that are predominantly responsible for their hydration or hydrolysis appear specialized for the role. They are now referred to as JHEH and JHE respectively. In the same book, Gill *et al.*<sup>30</sup> demonstrated that a potent juvenile hormone mimic was degraded in insects largely by epoxide hydration, and this report was also the first demonstration of the mammalian soluble epoxide hydrolase. It is also interesting that both of the major pathways of degradation of JH by insects involved proteins in the  $\alpha/\beta$ -hydrolase fold family, since both esterases and epoxide hydrolases have the same fold and fundamental catalytic mechanism. Several review papers have emphasized the degradation of juvenile hormone, but the diversity of insect species makes it difficult to draw generalized conclusions beyond the fact that in many species degradation of the hormone appears important in regulatory biology just as does biosynthesis, and that JHEH and JHE are often important routes of metabolism.<sup>31–34</sup>

In a landmark paper in *Nature*,<sup>10</sup> Brooks described the hydration of the juvenile hormone epoxide as a possible target for the development of pest control agents, and, based on his earlier work with the inhibition of the hydration of HEOM as a model substrate,<sup>3</sup> attempted to inhibit the degradation of JH with compounds optimized as inhibitors of EH action on HEOM in the fly *Caliphora erythrocephala* (Meigen) and the beetle *Tenebrio molitor* L. This work was also significant because he extended *in vitro* inhibition to *in vivo* bioassays. The EH inhibitors clearly synergized HEOM, but only ‘promising’ activity was observed with morphogenic assays of the inhibitors or as synergists of JH and juvenoids (JH mimics). Possibly, the work was of limited success because it was based on the assumption from competitive inhibition studies that HEOM and JH are hydrated by the same epoxide hydrolase. Such assumptions based on competitive inhibition of alternate substrates were common in the EH field,<sup>35</sup> but the assumption is quite dangerous, of course. As at the time this work was done there was no radioactive JH available, no LC-MS and GLC analysis of JH and its diol was very difficult, there was little other choice but competitive inhibition studies for analysis. It speaks poorly of research support in insect biology/physiology that, over 35 years later, it is still not known if Brooks’ assumption that HEOM EH is coincident with JHEH was correct with flies or beetles. It turned out not to be correct in the moth larvae (Lepidoptera) that have been examined. Of course, this *Nature* publication<sup>10</sup> speaks very well of Gerry Brooks’ insight. He first saw that targeting JH degradation has potential for pest control, and was the first in the field to try synergizing JH with JHEH inhibitors; and, of course, the inhibitors he found in his later studies predated the current best inhibitors known for JHEH.<sup>13,14</sup>

In the early 1970s, Mike Slade from Zoécon joined forces with Gerry Brooks in a long-term collaboration with Chris Wilkinson at Cornell. In 1976 they published an excellent paper comparing the epoxide hydration and its inhibition in three diverse insect species and also with the inhibition of the mammalian epoxide hydrolase.<sup>13</sup> This study was significant for many reasons. One was in showing differences between the mammalian and insect enzymes. They demonstrated the majority of the EH activity to be microsomal in the guinea pig, armyworm, blowfly and cockroach. They found weak EH inhibition by some alternative pharmacophores including methylene dioxyphenyl, propynyl ether, 1,2,3-benzothiadiazole and imidazole. The latter led to testing imidazoles to inhibit EH in biosynthetic preparations of cockroach *corpora allata* and finding that some imidazoles were powerful inhibitors of JH biosynthesis.<sup>36</sup> This led to subsequent excellent work by several laboratories on chemical inhibition of JH biosynthesis. Of particular note here, the authors found that the JHEH of the armyworm had a pH optimum of 7.8, while HEOM hydration was 9.0, and that the inhibition profiles with both substrates were different. They thus raised cautions about the use of HEOM as a surrogate substrate for JHEH, which, of course, should be generally applied to surrogates. They also cautioned that their data did not fully support the earlier interesting hypothesis of Slade and Wilkinson<sup>37</sup> that some juvenoids act by stabilizing JH, and supported the work of Solomon and Walker<sup>38</sup> to the contrary. This work was followed with HEOM hydration in the blowfly and yellow mealworm beetle, which reported glycidyl ethers, among other compounds, as micromolar inhibitors, and again reported 1,1,1-trichloropropene oxide (TCPO) as an inhibitor of EHs from mammals and insects. It is interesting to note, in retrospect, that these papers focusing on insects led to the best studies on the inhibition of the mammalian EHs in the literature of that time.<sup>14</sup> For example, the common alternative substrate sometimes used to inhibit the mammalian microsomal epoxide hydrolase, TCPO, was also a good inhibitor of the insect enzymes. In an excellent follow-up to these studies, Mullin and Wilkinson<sup>39,40</sup> reported the purification and characterization of the HEOM EH from the armyworm, showing that it was different from the enzyme metabolizing the JH epoxide. At the other end of the juvenile hormone, inhibition of JHE was demonstrated to have profound biological effects in insects quite early, using the chemical knockout approach advocated for JHEH by Gerry Brooks.<sup>41</sup> In fact, inhibition of JHE in *Manduca sexta* L. can result in massive larvae, while injection or transgenic expression of JHE can result in tiny black larvae,<sup>42,43</sup> and, in the silk worm, *Bombyx mori* L., even in precocious development.<sup>44</sup> However, similar experiments still have not been run with JHEH. Hopefully, either potent inhibitors will be developed or transgenic methods will

allow the testing of the hypothesis that JHEH is critical in insect developmental biology.

As referenced in several reviews, there is continuing interest in JHEH, but, of course, at a far lower level than the interest in the corresponding mammalian enzymes. JHEH generates the corresponding JH diol (Fig. 2), and there are periodic reports of some activity associated with this dihydroxy metabolite. Probably of more importance, the JH diol can be rapidly conjugated for excretion and it cannot be reconverted to biologically active JH.<sup>45</sup> As covered in several reviews, JHEH has been monitored in several model insects through development, with the lepidopterous larvae of *Helicoverpa zea* (Boddie), *Heliothis virescens* (Fabricus), *Manduca sexta*, *Bombyx mori* and *Trichoplusia ni* (Hübner) probably studied in most detail (see Khalil *et al.*<sup>46</sup> and references therein).

Based in part on the discovery of glycidyl ethers as inhibitors of EH, chalcone oxides were reported as potent inhibitors of the mammalian EH,<sup>47</sup> and Dietze and associates<sup>48,49</sup> reported that phenyl-glycidols were potent irreversible inhibitors as well. With regard to inhibition of JHEH, the work by Roe and associates with *T. ni* has been the most sustained. For example, by placing the glycidol functionality in a simple methyl ester where it corresponded to the 10,11 position of JH, a potent inhibitor of the JHEH of *T. ni* was produced. Similarly, by making a simple epoxy ester in much the same way as earlier work by Hafferl and Gilbert (unpublished), a good competitive substrate was synthesized.<sup>50</sup> A rapid screen using definitive JHEH is, of course, an important tool for developing JHEH inhibitors. The basic biology expanding on the early work of Mullin, Wilkinson and others to characterize how many JHEHs are involved in insect biology is still wanting. However, the cloning, characterization and expression of JHEH first from *M. sexta*,<sup>51,52</sup> *B. mori*<sup>53</sup> and *T. ni*<sup>54,55</sup> certainly helps with structural optimization, and hopefully target enzymes soon will become available from a variety of model species.<sup>56</sup> For example, using the recombinant JHEH from *M. sexta*, powerful JHEH inhibitors were developed<sup>57</sup> on the basis of the amide and urea pharmacophore optimized for the microsomal and soluble epoxide hydrolases.<sup>58,59</sup> Although these compounds offer metabolic stability, none of the JHEH inhibitors developed to date is sufficiently powerful to test the original hypothesis advanced by Gerry Brooks in his 1973 paper.<sup>10</sup> Hopefully, novel central pharmacophores like the ureas and amides when placed in a JH-like backbone will prove useful tools for future insect endocrinologists.

### 2.3 Epoxide hydrolases in pheromone metabolism

With regard to juvenile hormone regulation, it was thought that, because metamorphosis occurs so rapidly, the control of the degradation of JH as well as its biosynthesis was likely important in the regulation of the JH titer. As discussed below, the same argument

was made for the mammalian soluble epoxide hydrolase and its local hormone substrates. It has long been assumed in insect chemical communication that rapid degradation of a pheromone following its initial detection is critical for the insect to orient in a dynamic way.<sup>60,61</sup> The trifluoroketones as inhibitors of antennal esterases that degrade pheromone esters represent the best *in vivo* demonstrations of the phenomenon.<sup>60</sup> An elegant approach extending from the early work of Brooks in this area was based on the pheromone of the gypsy moth (*Lymantria dispar* L.) which has an epoxide on a hydrocarbon backbone. Graham and Prestwich<sup>62</sup> placed alpha fluorines and hydroxyl and carbonyl compounds to obtain more stable pheromones. Although Gerry Brooks did not emphasize pheromone chemistry in his early work on epoxide hydrolases, this represents yet another route by which his work has benefited other fields. Since a variety of xenobiotic metabolizing enzymes, including the microsomal epoxide hydrolase, are at high titers in nasal mucosa, it is a matter of speculation whether, in both insects and mammals, these enzymes could represent a first defense against xenobiotics in an exposed tissue, but also a mechanism by which habituation to odor molecules is avoided.<sup>63</sup>

## 3 MAMMALIAN MICROSOMAL EPOXIDE HYDROLASE (mEH)

The mammalian microsomal epoxide hydrolase certainly is the best known of the EH enzymes among pharmacologists and toxicologists because of its role in the metabolism of a number of potent mutagens, carcinogens and other xenobiotics. It is commonly referred to as mEH to distinguish it from the very distantly related soluble epoxide hydrolase or sEH.

### 3.1 History

Brooks' early work on epoxide hydrolysis led to the first comprehensive article on the mammalian mEH enzymatic reaction. Although epoxide hydration had been discussed by a variety of scientists, this was the first article to focus on the enzyme in the title and in particular to focus on its role in the metabolism of pesticides.<sup>3</sup> Over the last quarter of a century, mEH has been the most studied of the EH enzymes (see Newman *et al.*<sup>64</sup> for a recent review).

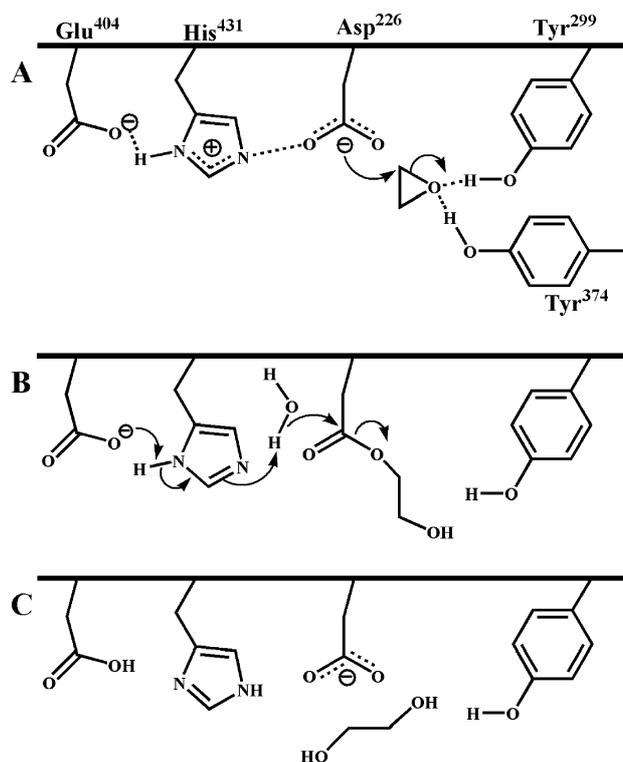
### 3.2 Biochemical characteristics

The microsomal epoxide hydrolase (mEH) is the product of the *EPXH1* gene situated on human chromosome 1. The mEH protein is made of 455-amino acid residues corresponding to a ~50 kDa protein, with a strongly hydrophobic transmembrane anchor of approximately 20 amino acid residues at the *N*-terminal. The *C*-terminal domain, which contains the catalytic residues, is homologous to a haloalkane dehalogenase. The mEH has been found in nearly all mammalian tissues that have been evaluated, but it is particularly concentrated in the liver, testis, lungs, heart

and adrenal glands. Intracellularly, mEH is mostly found attached to the smooth endoplasmic reticulum; however, there are a few reports showing it to be associated with the nuclear and plasma membranes.

Since its discovery, mEH has become recognized as a key enzyme in the metabolism of numerous environmental contaminants, including epoxides of polyaromatic hydrocarbons, and of drugs, such as 1,3-butadiene, phenantoin, carbamazepine and benzo[ $\alpha$ ]pyrene.<sup>35,65</sup> Early studies using a microsomal preparation and competitive substrates suggested that mEH prefers mono- and *cis*-1,2-disubstituted epoxides, while *gem*-di-, *trans*-di-, tri- and tetrasubstituted epoxides are low-turnover substrates or inhibitors.<sup>35,66</sup> A few endogenous substrates have been reported for mEH. In particular, androstene oxide (16 $\alpha$ , 17 $\alpha$  epoxyandrost-3-one) and estroside (epoxyestratrienol) were reported as endogenous substrates of mEH.<sup>64</sup> While epoxy fatty acids such as epoxystearic acid are relatively poor substrates for mEH compared with sEH (see Section 4), the former enzyme hydrolyzes this compound with a high enantioselectivity, while the latter does not. In addition, epoxide-containing glycerol-phospholipids are poor substrates for mEH.<sup>67</sup>

In their early work on mEH, Gerry Brooks and colleagues demonstrated that the product diols resulted from *trans* hydration of the epoxide substrate and were chemically identical to synthetic diol.<sup>3</sup> Although the stereochemistry of hydration did not absolutely define the catalytic mechanism, it eliminated many possible mechanisms resulting in *cis* diols. While establishing early on that the mEH mechanism is through a general nucleophilic attack on the carbon backbone of the epoxide, it took two decades to understand the catalytic mechanism of the mEH (see Morisseau and Hammock<sup>68</sup> for a recent review on the EH mechanisms). In a first step, the epoxide is polarized by two tyrosine residues (299 and 374), which hydrogen-bond with the epoxide oxygen (Fig. 3A). At the same time, the nucleophilic carboxylic acid of Asp<sup>226</sup>, present on the side opposite the catalytic cavity to the tyrosines, makes a backside attack on the epoxide, usually at the least sterically hindered and most reactive carbon. The nucleophilic acid is oriented and activated by His<sup>431</sup> and a second carboxylic amino acid (Glu<sup>404</sup>). The opening of the epoxide results in an ester bond between the enzyme carboxylic acid and one alcohol functionality of the diol. This is termed the hydroxyl-alkyl-enzyme intermediate (Fig. 3B). In a second step, the histidine moves far enough from the nucleophilic acid (now ester) to allow a water molecule to be activated by the acid-histidine pair (Fig. 3B). This very basic water attacks the carbonyl of the ester, releasing the diol product and the original enzyme (Fig. 3C).



**Figure 3.** Proposed mechanism for the human microsomal epoxide hydrolase: A, formation of a hydroxyl-alkyl-enzyme intermediate; B, hydrolysis of the intermediate to release C – the diol product and the original enzyme.

### 3.3 Biological roles

Epoxides are highly strained three-membered cyclic ethers. They range in reactivity from compounds such as dieldrin and heptachlor epoxide, which are environmentally persistent and very resistant to both enzymatic and chemical hydration, to epoxides that are so unstable and reactive that they may only exist on the pages of toxicology textbooks. Among the reactive epoxides are the ones that are metabolically generated from polycyclic aromatic hydrocarbon pollutants or aflatoxin B<sub>1</sub>, a mycotoxin.<sup>69,70</sup> These epoxide metabolites are largely responsible for mutagenesis and carcinogenesis resulting from exposure to the xenobiotic olefins.<sup>71</sup> Luckily, most epoxides are intermediate in reactivity; they are neither so stable as to be persistent nor so reactive that they alkylate proteins and nucleic acids. Epoxides can be degraded chemically or enzymatically to either glutathione conjugates or 1,2-diols or glycols.<sup>3,7,65,72–74</sup> This latter reaction always results in a dramatic increase in polarity, the generation of sites for enzymatic conjugation and generally a reduction in reactivity and toxicity.

Since the early work of Brooks and others,<sup>3,7</sup> mEH has been recognized as a key enzyme in the hepatic metabolism of numerous environmental contaminants.<sup>35,65</sup> The mEH is also involved in the extrahepatic metabolism of these agents, such as pulmonary naphthalene metabolism.<sup>75</sup> Because, in most cases, the resulting diols are less mutagenic or carcinogenic than the starting epoxide, mEH

has overall a cytoprotective role by detoxifying xenobiotics. The protective role of mEH from xenobiotics was illustrated in the case of a man with a defect in mEH expression, suffering from acute and severe phenytoin toxicity.<sup>76</sup> However, in the case of some polyaromatic compounds, such as benzo[ $\alpha$ ]pyrene or 7,12-dimethylbenz[ $\alpha$ ]anthracene (DMBA), dihydrodiol formation can stabilize the molecule after the formation of a second epoxide, yielding highly mutagenic 'diol epoxides' (Fig. 4) that can alkylate DNA while resisting further metabolism by mEH.<sup>77</sup> The procarcinogenic role of mEH was illustrated in mEH knockout mice that were less sensitive to the carcinogenic activity of DMBA than control mice.<sup>78</sup> Thus, inhibition of the mEH enzyme could be viewed as either enhancing or reducing toxicity, depending on the xenobiotic involved.

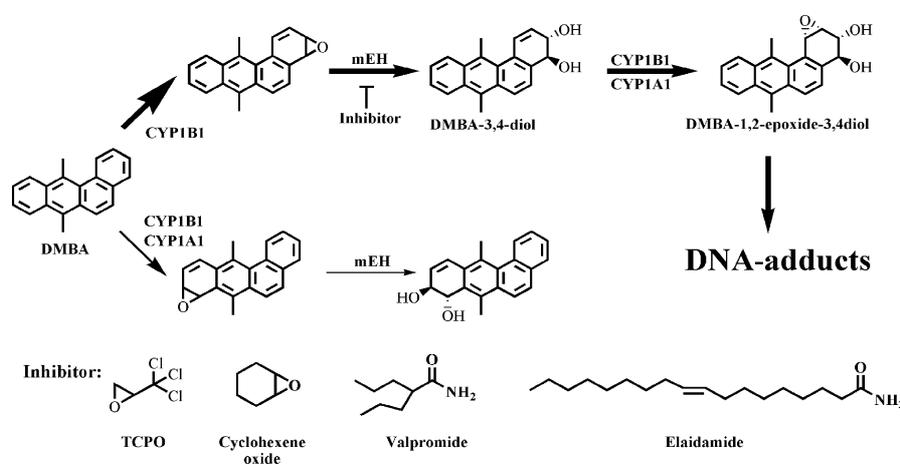
In spite of the fact that mEH knockout mice do not present any obvious phenotype, there are several new lines of evidence suggesting an endogenous role for this enzyme. A potential role of mEH in sexual development is supported by the observations that several epoxy steroids are very good mEH substrates and mEH is well expressed in ovaries and follicle cells, and it is reported that mEH is an apparent subunit of the anti-estrogen binding site.<sup>64</sup> Because some of these epoxy steroids may be toxic, mEH may be important in cellular protection from steroid metabolites, as it is in the metabolism of epoxidized xenobiotics. Over the last decade, mEH has also been described as mediating the transport of bile acid in the liver.<sup>79</sup> The mechanism by which mEH participates in this transport is not known. Potent mEH inhibitors could provide new tools for understanding better the multiple roles of this enzyme.

### 3.4 Inhibitors

In their work on cyclodiene epoxide metabolism in insect and mammals, Brooks and colleagues also initiated studies on inhibition of the mEH and emphasized

the use of chemical inhibitors to understand the biological role of an enzyme.<sup>10–14</sup> The first inhibitors discovered for the mEH were epoxide-containing compounds such as 1,1,1-trichloro-2,3-epoxypropane (TCPO) or cyclohexene oxide (Fig. 4).<sup>80</sup> However, most of these compounds are in fact substrates of the corresponding EH with a relatively low turnover that give only a transient inhibition *in vitro* and are inefficient in cell cultures and *in vivo*.<sup>81</sup> One of the best inhibitors of mEH is TCPO, and it is in fact turned over very quickly but outcompetes many substrates for the enzyme for a brief time. Around two decades ago, valpromide and progabide were reported to inhibit mEH with  $K_I$  values in the low micromolar range.<sup>82,83</sup> These compounds are effective mEH inhibitors both *in vitro* and *in vivo* because progabide or valpromide treatments increase phenytoin or carbamazepine toxicity due to epoxide accumulation.<sup>84</sup> The anticonvulsant properties of these compounds could cause undesirable secondary effects in experimental systems if used as mEH inhibitors, but other unsubstituted amides could be used.<sup>85</sup> Recently, primary ureas, amides and amines have been described as mEH inhibitors.<sup>59</sup> Elaidamide, the most potent inhibitor obtained so far, has a mix of competitive and non-competitive inhibition kinetics with a  $K_I$  of 70 nM. It is efficient *in vitro*; however, its fast turnover by amidases limits its use *in vivo*, underlying the need for new potent and stable mEH inhibitors as pharmacological probes.

The fact that valpromide or progabide treatments increase the toxicity of carbamazepine or phenytoin<sup>84</sup> underlines the risk associated with the therapeutic inhibition of mEH. However, this could not be generalized to all the substrates of mEH. For example, in the case of polyaromatic air pollutants such as benzo[ $\alpha$ ]pyrene or 7,12-dimethylbenz[ $\alpha$ ]anthracene, the action of mEH leads to more mutagenic and toxic metabolites (Fig. 4).<sup>77,78</sup> Thus, in this



**Figure 4.** Metabolism of DMBA leading to the formation of DNA adducts,<sup>77</sup> and structure of common mEH inhibitors. So-called arene oxides can be potent mutagens because the polycyclic nucleus intercalates into DNA and the epoxide electrophile reacts covalently with DNA. Diol epoxides are often more stable than the corresponding epoxides, possibly by inhibiting mEH, and can be potent carcinogens. Alternative substrates like TCPO and cyclohexene oxide have been used as mEH inhibitors. Some amide drugs such as valpromide are inhibitors as well. Medicinal chemical approaches have led to the most powerful inhibitors found to date, such as elaidamide, which may also have endogenous roles.

case the therapeutic inhibition of mEH will be beneficial. Furthermore, the genotoxicity of 1,3-butadiene, an important industrial chemical and common air pollutant, was found to be inversely correlated with predicted *in vivo* mEH activity.<sup>86</sup> For this compound, also, therapeutic inhibition of mEH will be beneficial. Early metabolism studies *in vitro* show that epoxides from xenobiotic olefins could be degraded by either glutathione-*S*-transferases or epoxide hydrolases.<sup>5,7,65,72–74</sup> However, the relative importance of each enzyme *in vivo* is not well understood. In the case of aflatoxin B1, kinetic measurements and experimentation in rodents clearly show the predominance of GSTs for the detoxification of this chemical.<sup>69,87</sup> Additionally, a recent microarray study suggests that resistance to the hepatotoxicity of bromobenzene in rats is mostly due to increased expression of GSTs and multidrug resistance protein 3.<sup>88</sup> Finally, mEH knockout mice are not susceptible to benzene-induced toxicity.<sup>89</sup> In conclusion, the metabolism of xenobiotics involves numerous enzymes and pathways: in most cases the inhibition of mEH will probably be compensated for by the action of other metabolizing enzymes, especially glutathione-*S*-transferase; in a few cases it will result in an increased toxicity; and in few cases it will result in decreased toxicity.

#### 4 SOLUBLE EPOXIDE HYDROLASE (sEH)

The mammalian soluble epoxide hydrolase was found a few years after the microsomal enzyme. Although it does degrade some xenobiotics, it appears that the major role of sEH is the metabolism of endogenous lipid chemical mediators. Of course, this observation raises the question as to whether any enzymes originally described as solely involved in xenobiotic metabolism in fact have an endogenous role in regulatory biology or intermediary metabolism.

##### 4.1 History

By the early 1970s the existence of the microsomal epoxide hydrolase was well established; thus, it was unexpected to find that the majority of the epoxide hydration of a terpenoid juvenile hormone mimic (juvenoid) occurred in the soluble or 100 000 × *g* supernatant fraction.<sup>30,90,91</sup> In fact, it was difficult to publish studies subsequent to the original papers of Gill during the 1970s because it was widely assumed that all epoxide hydrolase activity was microsomal. Several studies<sup>92–94</sup> finally established that the soluble enzyme had been overlooked by previous researchers:

(a) Because styrene oxide was used as a surrogate substrate for all mammalian EH studies. Styrene oxide is a difficult substrate to use because of its volatility, tendency to polymerize, background chemical hydrolysis and reaction with protein thiols. It is also a very poor substrate for the mammalian sEH.<sup>66</sup>

(b) Because early studies showed that the mEH has a basic pH optimum,<sup>3,9,80,81</sup> and, as styrene oxide is more stable at such pHs, the soluble fraction of tissues was often examined only at basic pHs, at which the sEH is largely inactive.<sup>66</sup>

(c) For reasons still not well understood, most rat strains have exceptionally low levels of the sEH, particularly in hepatic tissues, yet the rat was the most common animal used in tests.<sup>9,80,81</sup>

The original workers in the EH field restricted their conclusions to statements like ‘the epoxide hydrolase activity on styrene oxide at basic pH in rat liver homogenates appears exclusively microsomal’. However, reviews based on these studies often made such general statements that it became widely assumed that all epoxide hydrolase activity was microsomal, when in fact the observation was dependent on the species, substrate and assay conditions. For example, with the right substrate and assay conditions, it can be observed that all epoxide hydrolase activity is soluble.<sup>94</sup> The clear conclusion is that the two enzymes have complementary substrate selectivity and both appear to be of biological importance.<sup>92,93</sup> The leukotriene A4 hydrolase is an important clinical target, as it generates the biologically active LTB4 diol; however, it clearly acts by the formation of a carbocation mechanism.<sup>95</sup> There is clearly a cholesterol epoxide hydrolase that probably is not an  $\alpha/\beta$  hydrolase fold enzyme,<sup>68</sup> and genetic analysis indicates that there are several genes with a sequence that potentially code for other epoxide hydrolases.

The history of the discovery of sEH has been discussed previously,<sup>92</sup> but an interesting side light to this discovery involves work stemming from Konrad Bloch’s laboratory on the biosynthesis of lanosterol and cholesterol. Tchen and Bloch<sup>96</sup> reported that squalene epoxide was an early product in the conversion of squalene to lanosterol. They found that the process was enzymatic and that it required molecular oxygen. A variety of hypotheses were advanced to explain this cyclization, but the involvement of squalene 2,3-oxide was advanced in parallel by two major research groups. These were the Corey laboratory at Harvard and the van Tamelen laboratory at Stanford. Once the original idea of an epoxide intermediate was formulated, a variety of experiments to test the hypothesis of its involvement became obvious. These experiments were carried out in parallel and almost simultaneously on each coast. The results from the two laboratories usually were almost identical, but a key experiment was showing how the replacement of the methyl groups of squalene oxide influenced the cyclization process. Here, the results differed, but they did not differ with the cyclization reaction. The Harvard group reported that, when the substituents on the terpene epoxide were monomethyl, monoethyl or unsubstituted, formation of the corresponding diol was a major side reaction,<sup>97</sup> while the Stanford group did not report the diol.<sup>98</sup> The results could be predicted

from current knowledge. Gill *et al.*<sup>90</sup> hypothesized that the research community overlooked sEH because it was selective for trisubstituted terpene oxide. Mumby and Hammock<sup>99</sup> later demonstrated that tetra- and trisubstituted epoxides (like squalene oxide) were substrates, but were turned over more slowly than di- and monosubstituted terpene epoxides. However, the mono- and 1,2-disubstituted terpenes used by the Harvard group were very rapidly turned over by the sEH. Although both the Harvard and Stanford groups used rat liver for initial studies, and rats have very low levels of hepatic sEH, the Harvard group used an 'S-9' preparation for some initial studies, based on earlier work of their colleagues Bucher and McGarrah,<sup>100</sup> while the Stanford group used a real or  $100\,000 \times g$  microsomal preparation. Thus, the Corey group can be credited for first reporting sEH activity, but this knowledge was lost in subsequent studies when they used true microsomal or solubilized microsomal oxidocyclase preparations.

#### 4.2 Characteristics of the soluble epoxide hydrolase

The sEH is widely distributed in numerous tissues in mammalian species, but it is in exceptionally high concentration in the liver and kidney, as would be expected for an enzyme involved in xenobiotic metabolism.<sup>64,65</sup> However, even in the kidney its distribution varies dramatically with cell type, and with many other organs it is in high concentration in specific cell types. Certainly, in hindsight, its tissue distribution can be rationalized with a role in the regulation of blood pressure and inflammation, and increasingly with analgesia and neural function. On a subcellular level it is in the cytosolic or soluble fraction, but in some cases it can be localized in the peroxisomes. The regulation of subcellular localization remains confusing, but it clearly has at least dual localization.<sup>101,102</sup> With most substrates the enzyme has a near-neutral pH optimum, and it is resistant to changes in ionic strength. The enzyme is encoded by *EPHX2*, a single gene with 12 exons, and to date differential exon splicing has only been found in murine ovary.<sup>103</sup> The sEH exists largely as an antiparallel homodimer composed of a 62.5 kDa protein. Each monomer is a product of a gene fusion with an *N*-terminal phosphatase of unknown biological role connected by a proline rich linker to a *C*-terminal  $\alpha/\beta$ -hydrolase fold enzyme responsible for the epoxide hydrolase activity.<sup>64</sup> The catalytic mechanism involves two steps, and is similar to the one described above for the mEH (Fig. 3). Firstly, there is an attack of a nucleophilic aspartic acid on the epoxide carbon to give a stable, covalent intermediate, followed by hydrolysis by activated water to give a diol product. The mechanism is homologous to that of lipases and acetylcholinesterase.<sup>68</sup> The regulation of the sEH is quite different from that of the mEH in mammals. Quite early on it was found that drugs like peroxisome-proliferating chemicals (PPAR alpha

ligands) are strong inducers of sEH in rodents, while classical xenobiotic inducers are not.<sup>104</sup> However, PPAR alpha responsive elements have not been found upstream of the human *EPHX2*.<sup>105</sup> PPAR alpha ligands include a variety of pharmaceuticals, industrial chemicals and pesticides, with the fibrate drugs being the most widely used. sEH levels are known to be altered by sex hormones, although responsive elements have not been found to be associated with the gene. Of great physiological significance, the peptide chemical mediator angiotensin II induces sEH activity in the kidney, the vascular endothelium and in a reporter system tying the regulation of blood pressure and inflammation by the arachidonate cascade to the rennin-angiotensin system.<sup>106</sup>

#### 4.3 Substrate selectivity and role in arachidonate metabolism

Since the early work of Brooks showed that squalene oxide was a poor substrate for mEH, and that sEH was discovered with the trisubstituted epoxide of insect juvenile hormone and a juvenile hormone mimic,<sup>47,49</sup> early workers in the field thought that the sEH was overlooked because of its unique substrate selectivity.<sup>91</sup> The finding that the sEH degraded mono- and 1,2-disubstituted epoxides at a much greater rate than trisubstituted epoxides was a surprise.<sup>107</sup> However, given this information, the hypothesis that fatty acid epoxides may be endogenous substrates followed immediately,<sup>108</sup> and arachidonate epoxides were considered as likely biologically active candidates.<sup>109,110</sup> It is now widely accepted that epoxides of arachidonic acid (epoxyeicosatrienoic acids, EETs) and a variety of other fatty acids, amides and esters are chemical mediators of numerous processes (Fig. 5).<sup>111</sup> The EETs are widely assumed to be a major component of the endothelium-derived hyperpolarizing factor (EDHF) which leads to vasorelaxation.<sup>110</sup> In insect endocrinology it is widely accepted that, for chemical mediators whose titers change quickly, regulation of degradation may be as important as biosynthesis. This certainly is the case with the biological inactivation of insect juvenile hormone by highly selective esterases and epoxide hydrolases (see Section 2 above). This concept was not so widely accepted in mammalian regulatory biology, so the demonstration of the role of the sEH in EET regulation awaited the development of potent inhibitors for the enzyme that were active *in vivo*.

#### 4.4 Inhibitors of the soluble epoxide hydrolase

By screening alternative substrates as a possible indicator of the substrate selectivity of the mEH, weak inhibitors of the enzyme such as cyclohexene oxide and triclopropene oxide were found.<sup>35,81</sup> However, Brooks and colleagues were the first to take a systematic approach to identifying inhibitors of mEH.<sup>10-14</sup> Numerous approaches were taken to develop suicide and transition-state inhibitors of the sEH. This work resulted in finding that some



#### 4.5 Development of pharmaceutical candidates based on sEHIs

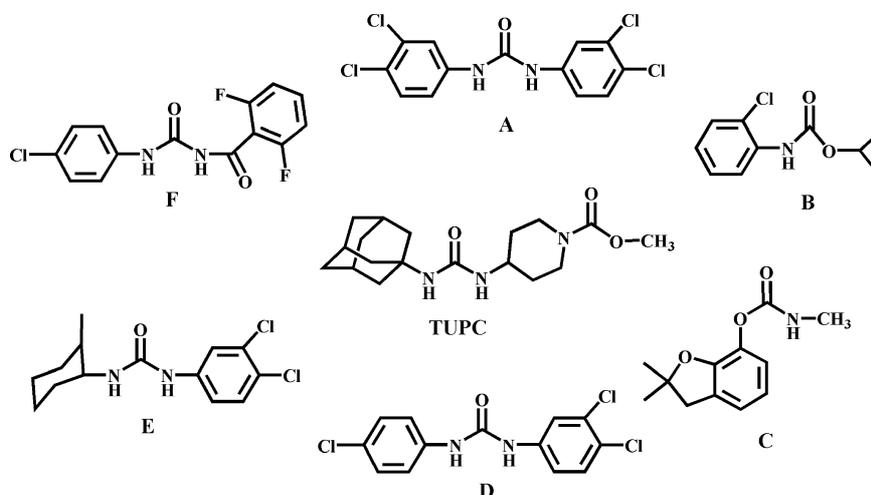
There are a number of interactive steps beyond the discovery of a simple sEH inhibitor leading to the validation of a therapeutic target. The original sEHIs, such as dicyclohexyl urea (Figs 5 and 6), are insoluble and have high melting points. This makes them very difficult to formulate in a way that biologists can use to test the role of the enzyme. The difficult physical properties of sEHIs undoubtedly led to a number of biologists becoming disillusioned with the role of the sEH in the regulation of EETs and other lipid epoxides. Similarly, the very rapid metabolism of EETs and related compounds by sEH resulted in their biological role being underestimated for many years. With the availability of sEHIs with improved physical properties, the role of the sEH and the EETs in reducing blood pressure, inflammation and pain have begun to be more widely accepted. However, even very potent sEHIs, such as AUDA,<sup>121</sup> remain very difficult to use and formulate. For any hope of a human pharmaceutical in the absence of exotic formulation, compounds with improved physical properties, good oral bioavailability and good pharmacokinetic properties are critical. Over several years, sEHIs were improved in the public sector firstly by driving physical properties and potency and then by screening for improved ADME (absorption, distribution, metabolism and excretion).<sup>122–124</sup> This has resulted in sEHIs (Figs 5 and 6) that are excellent probes, that validate sEH as a target for drug development and that can serve as leads for the pharmaceutical industry to improve the materials further. High-throughput assays based on recombinant enzymes make it possible to screen a broad chemical space for novel pharmacophores that act on sEH. Even the public-domain sEHIs represent a new mechanism of action for treating a variety of diseases and are as active as, or

more active than, commercial antihypertensives, anti-inflammatories and analgesics.<sup>125</sup> A new human pharmaceutical target where the catalytic activity was originally discovered during the investigation of a cyclodiene insecticide, the biology based on insights from insect endocrinology and the inhibitor structure derived from herbicide chemistry is an illustration of the integrated nature of Gerry Brooks' research. This work can be traced back not only to the original descriptions of the epoxide hydrolase enzyme by Brooks but also to his pioneering work on the inhibition of epoxide hydrolase.

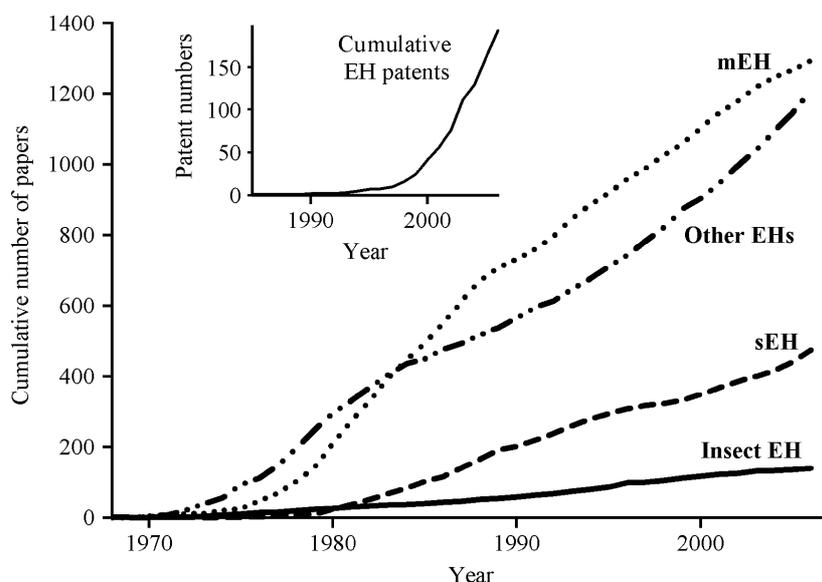
#### 5 EH IN OTHER SYSTEMS

Since the discovery of epoxide hydrolase activity four decades ago,<sup>3,7</sup> epoxide hydrolases have been found in every organism tested, from microorganisms to plants and animals. While the biological roles of EHs in insects and mammals have been, and are being, studied (see above), their role in other organisms is less well understood.

EHs have been characterized and cloned from at least a dozen plants (see Newman *et al.*<sup>64</sup> for a recent review). Plant EHs have been isolated from, or localized in, different tissues of plants. However, tissue distribution is quite variable from plant to plant, which underlines the overall lack of knowledge of plant EHs. As with the mammalian soluble EHs, plant soluble EHs are found primarily in the cytosol. There is clear evidence that plants contain multiple epoxide hydrolase isoforms. Several isoforms have been isolated in soybean, cress and potato, while unique constitutive and infection-induced forms have been suggested in tobacco.<sup>126</sup> Early work by Kolattukudy and collaborators suggests a role in cuticle formation, which is supported by the observation that epoxy fatty acids are good substrates for plant EH.<sup>127,128</sup> In addition, EH expression is increased in response to stress and pathogens in plants.<sup>126,129</sup> While the



**Figure 6.** Intellectual origin of powerful sEH inhibitors from pesticide and industrial chemicals. Powerful sEH inhibitors like TUPC came from basic structures such as dicyclohexyl urea (Fig. 5) and related structures including the diuron impurity A, chlorpropham B, carbofuran C, triclocarban D, siduron E and diflubenzuron F.



**Figure 7.** EH publications and patents from 1968 to 2006. Data were obtained and analyzed using SciFinder® for Scholar version 2006. Work on the mEH predated that on the sEH by approximately a decade.

mechanism is still unknown, there are indications that plant EHs have a role in regulating jasmonate signaling during periods of host response to attack by pathogens or insects. Intriguingly, the ability of certain plant EHs enantioselectively to hydrolyze some epoxides and/or to produce one diol enantiomer from a racemic epoxide has attracted the interest of organic chemists for the production of enantiopure compounds.<sup>128,130</sup>

For microorganisms, EHs seem important in the catabolism of specific natural carbon sources as well as environmental contaminants.<sup>131,132</sup> However, microbial EHs are mainly studied for their potential uses in chiral chemistry (see Smit and Labuschagne<sup>133</sup> for a recent review). Interestingly, in his seminal paper,<sup>3</sup> Brooks reported that the hydration of asymmetrical epoxides is stereoselective, with mainly only one enantiomer hydrolyzed, paving the way for the use of EHs for enantiomeric biocatalysis. While enantioselective reactions have been described for numerous epoxides and EHs, there are to date only two EHs commercially available as biocatalysts: one from *Aspergillus niger* (van Tieghem)<sup>134</sup> and one from a *Rhodococcus*.<sup>135</sup> Further, there is not yet any published industrial process using an EH. While most EHs from microorganisms have a mechanism similar to the one described above (Fig. 3), the limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* was recently found to have a one-step general-based-catalyzed direct hydration of the epoxide without the formation of a covalent intermediate.<sup>136</sup>

## 6 CONCLUSIONS

From his seminal work,<sup>3</sup> Dr Brooks opened the field of epoxide hydrolase research in many domains including xenobiotic metabolism, epoxides as chemical mediators in insect development, possible use in bioremediation and targets for improving human

health, as well as asymmetric organic synthesis. Over the past four decades, the EH field has generated more than 3000 research articles (Fig. 7). While most of the research has focused on mammalian enzymes (mEH and sEH), especially xenobiotic metabolism through mEH, there is a large body of knowledge on EHs from other organisms. As a result of this wealth of scientific knowledge, over the past decade around 200 patents have been filed, and these are likely to pave the way for beneficial technologies based on the epoxide hydrolases. A direct impact of the pioneering work of Gerry Brooks and his colleagues is illustrated by the small-molecule inhibitors of sEH that are entering phase-1 clinical trials this year for the treatment of high blood pressure, roughly 40 years after Gerry initiated his studies on epoxide hydration.

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