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Research Article

IN VITRO SOLUBLE EPOXIDE HYDROLASE ENZYME INHIBITORY ACTIVITY OF SOME NOVEL CHALCONE DERIVATIVES

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Abstract
Objective Soluble epoxide hydrolase **(**sEH) belongs to the α/β -hydrolase superfamily, a subclass of α/β proteins. Chalcones are chemical compounds that show hopeful obliging efficacy in controlling numerous diseases. The main objective of the study is to evaluate the sEH inhibitory activity of some synthesized chalcone derivatives and identification of its mode of inhibition.
Methods Four different chalcone derivatives (PC-1 to PC-4) were selected for synthesis by Claisen-Schmidt method. The *in vitro* sEH inhibitory activity was performed for the synthesized compounds by fluorimetric assay. The percentage of sEH activity and IC_{50} values were calculated for the synthesized compounds. Dissociation constant were determined by following the method described by Lineweaver-Burks plot.
Results and Conclusions The IC_{50} value obtained for PC-1, PC-2, PC-3, and PC-4 were found to be 0.8213 µg/mL, 2.64 µg/mL, 0.2490 µg/mL and 0.5238 µg/mL respectively. The order of potency (IC_{50}) of the chalcone and chalcone oxide in sEH inhibition assay was PC-3 > PC-4 > PC-1 >PC-2. All the compounds (PC-1, PC-2, PC-3) showed mixed type of inhibition except PC-4 which showed non-competitive type of inhibition. Further *in vivo* studies are to be carried out for these compounds to confirm their activity and explore the mechanism by which these compounds act and rationalize their use.
Keywords: Inhibitory concentration; Enzyme kinetics; Mode of inhibition; Dissociation constant

1. Introduction

Epoxide hydrolases are a group of xenobiotic enzyme that catalyzes the hydrolysis of epoxides by the addition of water molecules to it thereby leading to the formation of corresponding diols. They are found ubiquitously in nature and are present in most living organisms like mammals, plants, insects and various microorganisms such as yeasts, filamentous fungi and bacteria[1, 2].
Soluble epoxide hydrolase (sEH), also known as cytosolic epoxide hydrolase is the primary enzyme responsible for the conversion of epoxyeicosatrienoic acids (EETs) to the corresponding dihydroxyeicosatrienoic acid (DHETs). It is a xenobiotic metabolizing enzyme that also participates in the metabolism of endogenously derived fatty acid epoxides. The incorporation of EET into phospholipids is also regulated by sEH. It catalyzes the hydrolysis of *trans*-substituted epoxides, as well as the other aliphatic epoxides derived from fatty acid metabolism. The prototypic substrate used to distinguish sEH activity from microsomal epoxide hydrolase (mEH) activity is *trans*-stilbene oxide, other more sensitive radiometric substrates include [14C] *cis*-9, 10-epoxystearic acid and [2-3H] *trans*-1,3-diphenyl-propene oxide. Several additional specific sEH substrates have been developed and characterized, including a series of epoxy esters and carbonate-derivatives that cyclize spontaneously upon hydrolysis of the epoxide, releasing an alcohol. These substrates, therefore, enable rapid spectrophotometric analysis of sEH activity without the use of radiolabeled probes. Several classes of soluble epoxide hydrolase inhibitors (sEHI) have been developed which includes the early chalcone oxide derivatives and *trans*-3-phenylglycidols and the more recent urea and

carbamates which are more potent with nanomolar range of inhibition. Heavy metals like cadmium (Cd^{+2}) and copper (Cu^{+2}) have been identified as competitive inhibitors of sEH[3].

sEH activity is widely distributed in mammalian tissues with the highest activity in tissues like liver, kidney, intestine, and vascular tissue. Lower levels of sEH are found in testes, lung, brain and spleen. Although sEH is predominately located in the cytosol, the enzymatic activity and protein are present in the peroxisomes[4]. Inhibition of the sEH leads to elevation of endogenous EET levels thereby inhibitors are considered to be a potential novel target for the treatment for renal, cardiovascular, and neurological disorders[5].

sEH belongs to the α/β -hydrolase superfamily, a subclass of α/β proteins[6]. It is a homodimer with each monomer consisting of two domains that have different enzymatic activities[7]. The carboxy-terminal (C terminal) domain is responsible for the sEH activity, whereas the amino-terminal (N – terminal) domain exhibits Mg^{+2}-dependent lipid phosphatase activity. Dihydroxy lipid phosphates and polyisoprenyl phosphates which aid in sterol biosynthesis are good substrates of phosphates. The two domains function independently of one another, and inhibition of one activity does not affect the function of the other. Potent inhibitors of the sEH activity are now available, and lipid sulfates and sulfonates are being developed as inhibitors of the lipid phosphatase activity[8].

Selective inhibition of sEH has led to increased levels of EETs resulting in the potentiation of their *in vivo* pharmacological effect and hence it has been an emerging target for pharmacological treatment of cardiovascular, renal and neurological diseases. sEHI developed are still under clinical trials[9]. One of the urea NH-groups donates the hydrogen atom and forms an enzyme-inhibitor complex with the carboxylate chain of Asp^{333} amino acid residue[10]. However the poor bioavailability has limited their action hence substitution were done on the 1,3- position with bulky groups such as adamantyl, cyclohexyl, alkyl or aryl groups, to increase the bioavailability[11].

Chalcones are chemical compounds that show hopeful obliging efficacy in controlling numerous diseases. Chemically they comprise an open chain flavonoid with a three carbon α, β-unsaturated carbonyl system. They act as precursors of flavonoids as well as isoflavonoids[12]. They are an important constituent, obtained from natural sources, found to be associated with several biological activities[13]. These are found abundant in edible plants, the most common chalcones are phloretin and its glucoside; phloridzin (phloretin-2'-O-glucose) (in apples), chalconaringenin (tomatoes) and arbutin (strawberry, bearberry, wheat, tea, coffee and red wine). Studies on the bioavailability of chalcones from food sources are limited but synthetic chalcones have been reported to possess wide range of biological properties, hence they are more preferred. Chalcones are also used to synthesize several derivatives like cyanopyridines, pyrazolines, isoxazoles, pyrimidines, with different heterocyclic ring system[14]. Current objective of the study is to evaluate the sEH inhibitory activity of some synthesized chalcone derivatives and identification of its mode of inhibition by enzyme kinetic study.

2. Materials and Methods

2.1 Chemicals required: Purified recombinant human soluble Epoxide Hydrolase enzyme and cyano (2-ethoxynaphthalen-6-yl) methyl (3-phenyloxiran-2-yl) methyl carbonate (CMNPC) from Cayman chemicals, USA; 6-methoxynaphthalene-2-carbaldehyde (MNC) and Bovine serum albumin purchased from Sigma Aldrich, USA. All other chemicals used in the study were commercially obtained and were of analytical grade.

2.2 Synthesis of chalcones and chalcone oxide: Four different chalcone derivatives were selected for synthesis and the synthesized compounds were subjected to *in vitro* sEH inhibitory activity. The synthesis was carried out by Claisen-Schmidt method with simple stirring in dark for 45 minutes in case of chalcones and 3 hours for chalcone oxides. The introduction of the side-chain was also done by simple stirring at 15˚C with the help of an ice bath.

2.3 In vitro sEH Inhibitory activity

Preparation of reagents

- **Buffer 25mM Tris-HCl containing 0.1 mg/ml bovine serum albumin (BSA) -** About 3.94 g of Tris HCl buffer was weighed and dissolved in minimum quantity of distilled water and the final volume was made up to 1000 mL and pH was adjusted to 7.0 with 1N sodium hydroxide.

Bovine serum albumin fraction V was added at a concentration of 5 mg per 50 mL of buffer just before use.

- **CMNPC 0.5 mM -** Initially the working substrate solution was prepared by dissolving 3.94 mg of CMNPC in 20 mL of DMSO. Later 40.89 mL Tris HCl buffer was mixed with 3.14 µL of 0.5 mM CMNPC.
- **sEH enzyme** (1.2 µg/mL)- About 25 µg of the enzyme solution was dissolved in 20.30 mL of the buffer such that the final concentration was 1.2 µg/mL.
- **Inhibitor solution-** A stock solution of all the inhibitor **(**0.12, 0.25, 0.50, 1, 2, 4 and 8 µg/mL**)** was prepared by dissolving the inhibitors in DMSO. From the stock solution various concentrations of the inhibitor were using DMSO prepared.

Procedure: About 742.57 µL of buffer was added to all the set of test tubes followed by 9.9 µL of DMSO in the control blank (B) and control total activity (T) tubes. About 9.9 µL of inhibitor solution (in DMSO) of various concentrations was added to the series of test tubes. As there might be a variation between the volumes of the test tubes, the assay was carried out in triplicate. Then the contents of the test tubes were mixed well. Later about 99 µL of buffer was added to the blank control (B) tubes. About 99 µL of 1.2 µg/ml of purified recombinant human sEH in buffer was added to the total activity (T) tubes and the test tubes containing the inhibitor (I).Then the contents of the tubes were mixed well for 10 sec and incubated for 5 min at 30˚C. Later 148.51 µL of working substrate solution was added to all the tubes (final concentration= 5 µM), the contents were mixed well and the fluorescence readings were recorded. The readings were taken at an excitation wavelength of 330 nm, an emission wavelength of 465 nm[15]. The average fluorescence of blank control (C), total activity (T) and inhibitor (I) values were calculated. The average fluorescence of the blank control was removed from the total activity and the inhibitor tubes. The percentage of sEH activity retained and IC_{50} values was calculated. The formula for calculating the percentage inhibition is:

Percentage inhibited = 100- [(FI of sample/FI of control) X 100]

Where, FI is the fluorescence intensity [15,16].

Enzyme Kinetics: Dissociation constant were determined by following the method described by Lineweaver-Burks plot. About 150 µL of enzyme (sEH) - buffer (Tris) solution (pH-7.0) was added to all the test tubes. Then 20 µL of inhibitor dissolved in DMSO was added to all the test tubes. The contents of the test tube were allowed to incubate at 30˚C for 5 minutes. Then the substrate was added into the tubes (50 µL) such that the final concentration was 0.5, 1, 2.5, 5.0, 7.5, 10.0, 15.0 µM. Then the fluorescence intensity was measured at an excitation wavelength of 330nm and an emission wavelength of 465nm. The V_{max} and K_m were determined using GraphPad Prism 5.0. A graph was plotted for the velocity against the concentration of the substrate and the type of inhibition was determined[17].

3. Results and Discussion

The synthesized chalcones and chalcone oxides chemical structures were shown in Fig.1. The various parameters such as IUPAC name, Molecular weight, Molecular formula, Percentage yield, Melting point and Retention factor (Rf) values for the synthesized compounds were tabulated (Table 1).

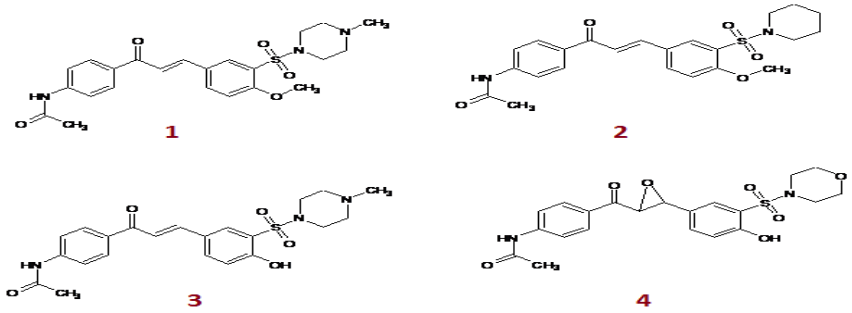

Fig. 1 Chemical structures of the synthesized compounds

Table 1. Parameters of synthesized compounds

Compound No	IUPAC Name	Molecular Weight	Molecular Formula	% yield	Melting point	Rf Value
1	[(4-acetamido)-(4'-methoxyl)-3'-(N-methylpiperazino)-sulphonyl)(2E)-1,3-diphenylprop-2-en-1-one]	457.54258	$C_{23}H_{27}N_3O_5S$	73%	197.4˚C	0.53
2	[((4-acetamido)-(4'methoxyl)-3'(piperidino)-sulphonyl)(2E)-1,3-diphenylprop-2-en-1-one]	442.52794	$C_{23}H_{26}N_2O_5S$	78%	207.4˚C	0.62
3	[((4-acetamido)-(4'-hydroxy)-3'-(N-methylpiperazino)-sulphonyl)(2E)-1,3-diphenylprop-2-en-1-one]	443.516	$C_{22}H_{25}N_3O_5S$	65%	167.5˚C	0.59
4	[((4-acetamido)-(4'-hydroxy)-3'-(morpholino)-sulphonyl)(2E)-1,3-diphenylprop-2-oxirane-1-one]	446.47358	$C_{21}H_{22}N_2O_7S$	65%	217.1˚C	0.67

The synthesized chalcones and chalcone oxides were tested for sEH enzyme inhibition studies by fluorometric method with CMNPC as the substrate. The test was carried out at various concentrations of 0.12, 0.25, 0.50, 1, 2, 4 and 8 µg/mL and the absorbance was measured at excitation wavelength of 330 nm and an emission wavelength of 465nm. The method was based upon the inhibition of sEH by the synthesized chalcone and chalcone oxides. sEH enzyme converts the substrate CMNPC, a non-flourescent substrate into 6-MNC, a fluorescent substrate whose emissivity is measured. The inhibition is dose dependent i.e., as the concentration of the compound increases the formation of 6-MNC was decreased with a resultant decrease in the fluorescence intensity confirming the inhibition of sEH. Of the four compounds PC-3 (i.e., chalcone moiety with acetamido at 4 position, OH at 4' position and sulphonyl –N-methyl piperazine substitution at 3' position showed the highest sEH inhibitory activity with a nanomolar inhibition constant (IC_{50}= 249nM).

In table 2, a dose dependent increase in the percentage of inhibition can be observed. The test compounds PC-1, PC-2, PC-3, PC-4 were found to inhibit 17.67%, 19.29%, 36.82%, 25.07% of sEH at 0.12 µg/mL respectively and an increase in inhibition to 80.51%, 71.14%, 96.13%, 91.82% at 8 µg/mL respectively. The IC_{50} value obtained for PC-1, PC-2, PC-3, and PC-4 were found to be 0.8213 µg/mL, 2.64 µg/mL, 0.2490 µg/mL and 0.5238 µg/mL respectively. The order of potency (IC_{50}) of the chalcone and chalcone oxide in sEH inhibition assay was PC-3 > PC-4 > PC-1 >PC-2. The results were found to be statistically significant and the $P < 0.005$ when compared between the compounds (One-way ANOVA).

Table 2. *In vitro* sEH inhibition results of synthesized chalcones and chalcone oxides by fluorimetric method

Concentration (µg/mL)	Percentage inhibition of sEH activity			
	PC-1	PC-2	PC-3	PC-4
0.12	17.67±0.36	19.29±0.55	36.82±0.52	25.07±0.46
0.25	28.13±0.46	26.38±0.45	50.01±0.32	38.39±0.26
0.50	37.33±0.63	32.19±0.52	59.89±0.60	49.22±0.81
1.00	57.73±0.28	38.44±0.46	68.88±0.19	61.50±0.52
2.00	66.76±0.29	46.43±0.59	77.97±0.48	73.04±0.54
4.00	74.87±0.72	57.22±0.19	85.92±0.32	84.66±0.47
8.00	80.51±0.45	71.14±0.13	96.13±0.49	91.82±0.52
IC_{50} (µg/mL)	**0.8213±0.64**	**2.64±0.39**	**0.2490±0.52**	**0.5238±0.69**

All determinations were done in triplicate and values are expressed as the mean ± SEM
The results were statistically significant P<0.05 (One-Way ANOVA)

The *in vitro* potency of the newly synthesized chalcone PC-3 was found to be in nanomolar concentration while the chalcones for which the sEH inhibition has been reported earlier were in micromolar concentration. This increase in potency for the chalcone nucleus may be due to the incorporation of an -NH and -CO groups at the 4' and 3' position, which bind to the nucleophile (Asp^{333}) through a hydrogen bond.

Enzyme kinetics is the rate of substrate utilization by the enzyme at varying concentrations of the substrate. The mechanism of the compound by which it inhibits the enzyme is known from the Lineweaver-Burk's plot. In competitive inhibition the inhibitor compete for the same active site as that of substrate and the increase in K_m value is seen but the V_{max} remains unchanged. In non-competitive inhibition, the inhibitor reacts with the binding site adjacent to that of the substrate and inhibits the enzyme action. Here the K_m is unchanged and the V_{max} is reduced.

The enzyme kinetic studies were performed for sEH enzyme for the synthesized chalcones at varying concentrations of the substrate. In mixed type of inhibition, the inhibitor can bind to both the free enzyme as well as the enzyme-substrate complex. Here both V_{max} and K_m obtained were different from the control values[18].

Enzyme kinetic studies helps to determine the type of inhibition (competitive, non-competitive or mixed type) produced by the inhibitors on the enzyme. The enzyme kinetic study for sEH enzyme was performed and various enzyme kinetic parameters such as K_m and V_{max} were determined by Michealis-Menten equation and the type of inhibition was determined by Lineweaver-Burk's plot using GraphPad Prism 5.0 (Fig. 2).

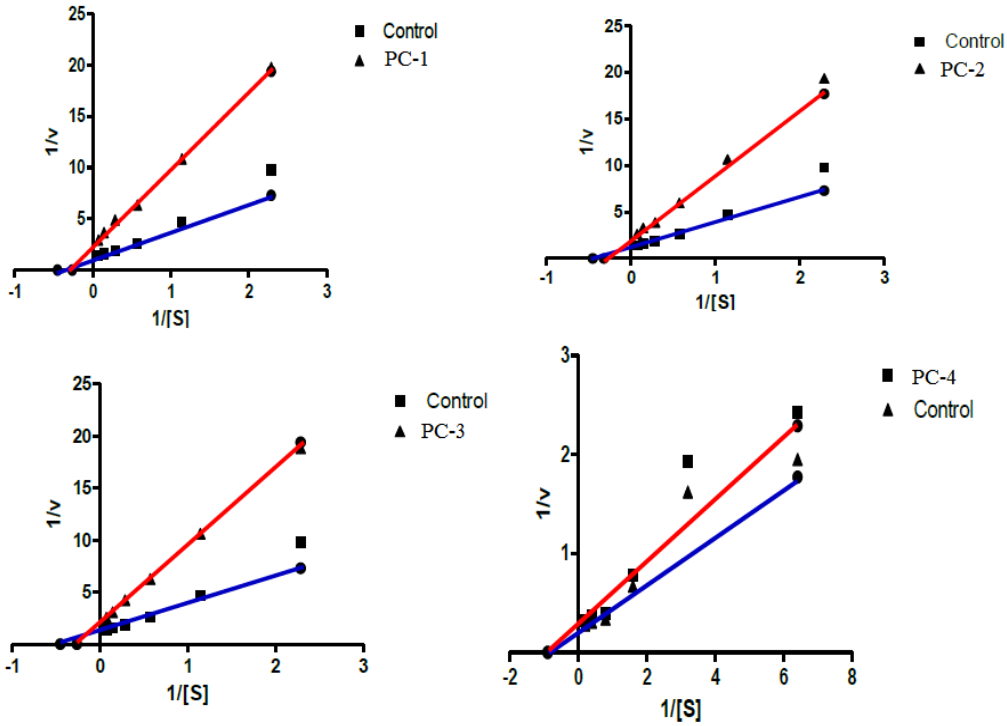

Fig. 2 Lineweaver-Burk's plot of synthesized compounds

The K_m values of the compound were found to be more when compared to the control. The K_m of the compound PC-1, PC-2, PC-3, PC-4 were found to be 3.02, 3.09, 3.90 and 1.063 respectively and the V_{max} value for the compounds were found to be 0.38, 0.45, 0.50 and 4.45 respectively. The values along with type of inhibition are provided in Table 3. All the compounds (PC-1, PC-2, PC-3) showed mixed type of inhibition except PC-4 which showed non-competitive type of inhibition. All the results were found to be statistically significant with $P<0.05$ (One-way ANOVA).

Table 3. Enzyme kinetics of soluble *epoxide hydrolase* enzyme

Compound code	K_m ± SEM (mM)	V_{max} ± SEM (dA/sec)	Type of inhibition
PC-1	3.02 ± 0.30	0.38 ± 0.01	Mixed
PC-2	3.09 ± 0.29	0.45 ± 0.01	Mixed
PC-3	3.90 ± 0.15	0.23 ±0.15	Mixed
PC-4	1.063 ± 0.46	4.45 ± 0.56	Non-competitive

All determinations were carried out in triplicate and values are expressed as the mean ± SEM
The results were statistically significant P<0.05 (One-Way ANOVA)

Conclusion

In conclusion, the sEH has been the focus in the field of lipid research at present as it is the most preferred approach to increase the level of EETs *in vivo* for eliciting its physiological actions. From the *in vitro* studies, it can be stated that all the synthesized compounds showed sEH inhibitory activity, especially PC-3 showed excellent sEH inhibitory activity. Further *in vivo* studies are to be carried out for these compounds to confirm their activity and explore the mechanism by which these compounds act and to rationalize their use.

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