CHAPTER 1

INTRODUCTION

1.1) Affinity capillary electrophoresis (ACE)

1.1.1) Capillary electrophoresis (CE)

Capillary electrophoresis (CE) is a modern analytical technique which permits rapid and efficient separations of charged components present in samples of small volumes. Separations are based on the differences in electrophoresis motilities of ions in electrophoresis media inside small capillaries [1-2]. One of the main advantages of CE is that it requires only simple instrumentation. It consists of a high-voltage power supply, two buffer reservoirs, a capillary and a detector (Fig. 1.1).

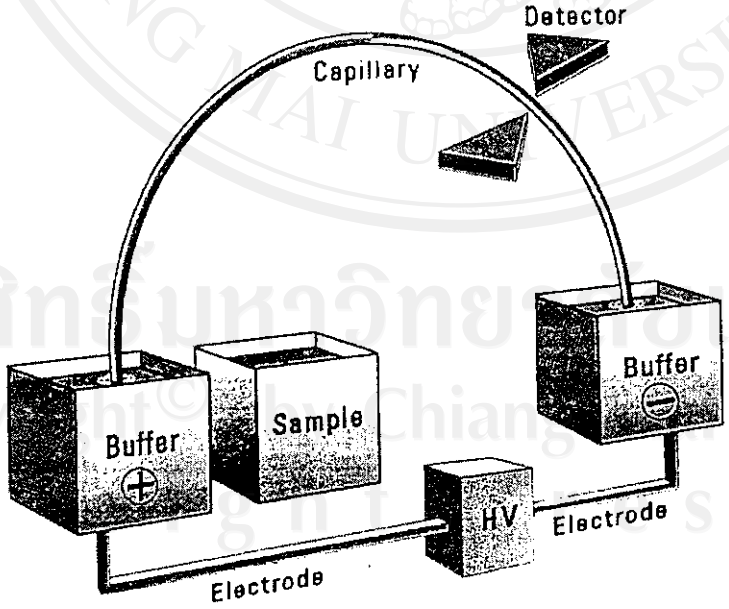


Figure 1.1 Schematic diagram of a system for capillary electrophoresis.

The basic instrumentation includes a separation capillary column dipped into the background electrolyte reservoirs equipped with platinum electrodes for the attachment of a high-voltage power source. Injection of samples onto the capillary column can be accomplished either by pressure drop to deliver a fixed volume or electromigration in which the applied electric field gradient transports the charged species onto the capillary column. After the introduction of high-voltage to the ends of the bare fused silica capillary column filled with electrophoresis buffer at physiological pH, the electroosmotic flow (EOF) transports the sample along the capillary column while maintaining a plug-flow geometry [3]. Monitoring of sample separation can be achieved utilizing a wide variety of detection methods. The mechanism of high-resolution separation has been well established with the observed migration time of the species dependent on its electrophoresis mobility and the velocity of the EOF.

Different modes of capillary electrophoresis separations can be performed using a standard CE instrument. The origins of the different modes of separation may be attributed to the fact that capillary electrophoresis has developed from a combination of many electrophoresis and chromatographic techniques. In general terms, it can be considered as the electrophoretic separation of a number of substances inside of a narrow tube. Even though most applications have been performed using liquids as the separation media, capillary electrophoresis techniques encompass separations in which the capillary contains electrophoresis gels, chromatographic packings or coatings. The distinct capillary electroseparation methods include: (A) Capillary zone electrophoresis (CZE); (B) Capillary gel electrophoresis (CGE); (C) Micellar electrokinetic capillary chromatography (MEKC); (D) Capillary electrochromatography (CEC) (Fig. 1.2).

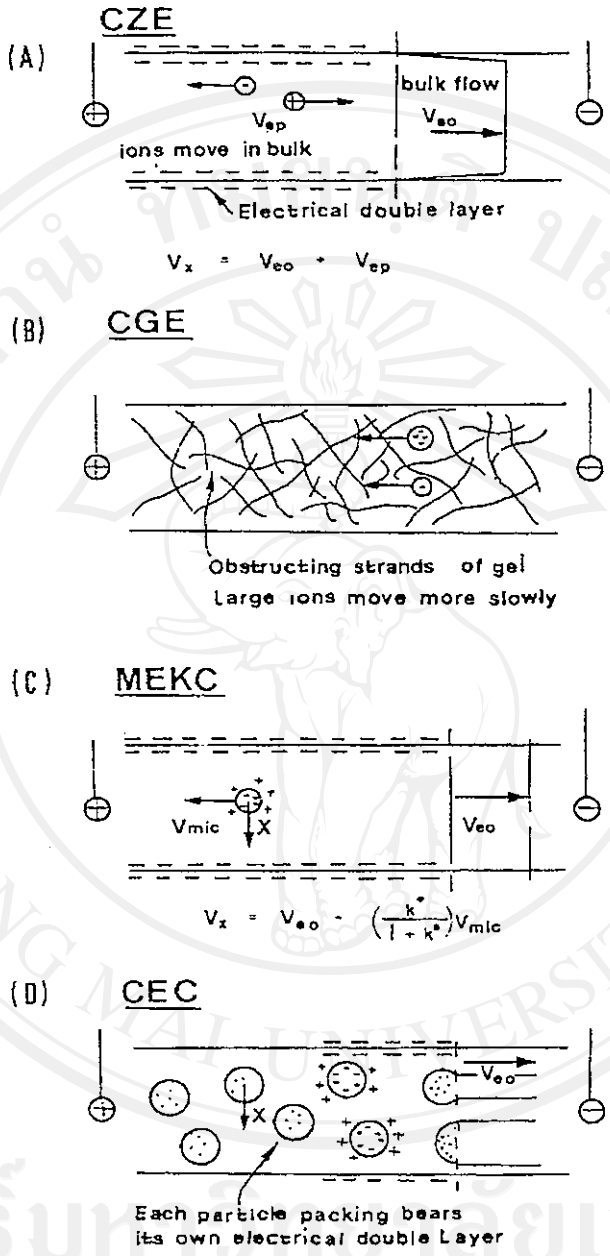


Figure 1.2 Diagramatic representation of (A) capillary zone electrophoresis (CZE), (B) capillary gel electrophoresis (CGE), (C) micellar electrokinetic capillary chromatography (MEKC), and (D)capillary electrochromatography (CEC). Vx is the linear migration velocity of the analyte X. Veo is the electroosmotic velocity, Vep is the electrophoretic velocity and k' is the phase capacity ratio.

Many biochemical applications using CE have been reported, including: (A) the discovery of dopamine in single lymphocytes of human cerebrospinal fluid implicating a role in immune activation [4]; (B) the DNA sequence determination of a template from the malaria genome [5]; (C) therapeutic monitoring of drugs such as anti-epileptics [6-8], anti-asthmatics[9,10], analgesics[11], antidepressants[12], benzodiazepines [13] and antitumor drugs [14-16]; (D) quantitative immunoassays by CE for antigens such as cortisol [17-18], morphine [19], digoxin [20], chloramphenicol [21] and angiotensin [22]. Pioneering studies by Karger [3,23], Grossman [24,25], Regnier [26,27], Novotny [28,29], Jorgenson [30,31] and others have shaped the rapid expansion of CE to commercialization where the development of versatile and inexpensive instrumentation makes CE routinely utilized. The CE emerged as a high resolutive separation technique, it is primarily limited by the particular detection method utilized. The most widely employed detection methods include ultraviolet-visible (UV-VIS) absorbance [32-34], fluorescence [35-40], and mass spectrum [41-47]. Most of biologically active compounds absorb within the range of 200-400 nm, commercially available CE instruments are all equipped with an UV-VIS detector.

1.1.2) Affinity capillary electrophoresis (ACE)

Affinity capillary electrophoresis (ACE) is a new procedure for studying protein-ligand interactions. Its potential applications include developing tight-binding drug candidates, screening libraries for lead compounds, characterizing the effective charges of proteins, and measuring enzymatic activities. Superior to other available techniques for studying binding interactions, ACE has recently demonstrated its value in the measurement of binding constants [48-50], estimation of kinetic rate constants [51],

determination of binding stoichiometries of receptor-ligand interactions [52] and combinatorial library screening in biochemical systems [53-55].

The overall attractive features of ACE include: (A) it provides an assessment of protein-ligand interactions using very small amounts of samples in a relatively short time; (B) it does not necessitate high protein purity or an accurate value of its concentration, since the binding constant is only based on electrophoresis mobility and shape of the peak; (C) it is applicable to the binding of several proteins to a given ligand in the same solution or vice versa; (D) it does not require the synthesis of radioactive or chromophoric ligands, although it does require the synthesis of a charged analog of the ligand; (E) it is capable of differentiating between protein conformations that bind ligand from conformations of the same protein that are denatured and do not bind ligand; (F) the commercial availability of automated instrumentation and the high reproducibility of data favor the use of this technique. A major limitation of ACE remains the tendency of proteins to adsorb onto the wall of uncoated capillary columns that can lead to poor separations and loss of material. Although this limitation becomes more pronounced when the pH of the electrophoresis buffer lies close to or lower than the isoelectric point of the protein, the problem can be resolved by utilizing coated capillaries [56,57].

In binding systems where receptor proteins associate tightly with ligands, the procedure of the measurement of binding constants is straightforward. Direct integration of the peak areas of the free and bound receptor protein allow a measurement of binding constants, provided that the free and bound species have different electrophoresis mobility.

1.1.3) Applications of ACE

1.1.3.1) Biosensors

A biosensor system based on the response of living cells was demonstrated that can detect specific components of a complex mixture fractionated by a microcolumn separation technique. This system uses ligand-receptor binding and signal-transduction pathways to biochemically amplify the presence of an analyte after electrophoretic separation.

Biosensors detect chemical species with high selectivity on the basis of molecular recognition rather than the physical properties of analytics. Many types of biosensing devices have been developed in the past 30 years, including enzyme electrodes, optical immunosensors, ligand-receptor amperometers and evanescent-wave probes [58-62]. Entire living cells also can be used as biosensors [63,64]. Whole-cell biosensors have two important advantages. First, many disparate chemical species can evoke a response from a single cell. Second, the recognition event for a component can be amplified by signal-transduction pathways so that measurable responses result from minute quantities of material [65]. When more than one component in a sample mixture elicits a response, the signal from a living cell often cannot be interpreted. The microcolumn separation technique of capillary electrophoresis is well suited for this purpose because of its physiologic compatibility, speed and high separation efficiency [66]. The ability of capillary separations to work with very small samples often makes it possible to analyze the components of individual cells without prohibitive dilution [67]. Fractionation of biological samples with many components has proved difficult for CE and a variety of important biological species are not easily detected with traditional measurement approaches [68](Fig.1.3).

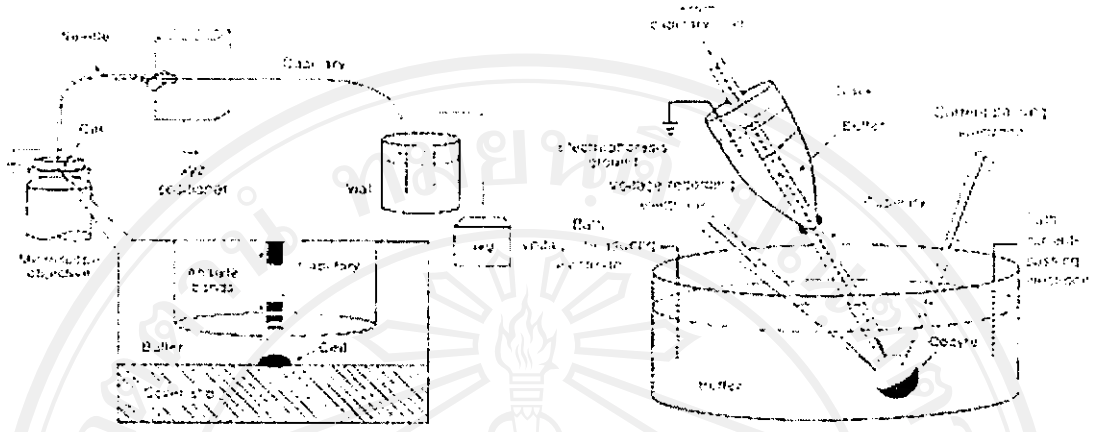


Figure 1.3 A capillary electrophoresis of single-cell biosensor (CE-SCB) systems.

1.1.3.2) Biomolecular recognition [69]

ACE is a new method for the studies of biomolecular recognitions. Its applications reported in the literature include chiral separation of racemic biomolecules, the measurement of binding constants, the estimation of kinetic on- and off- rate constants, the determination of binding stoichiometries, a useful tool in examining electrostatic interactions, the estimation of effective charges and molecular weights of proteins, the characterization of enzymatic activities and library screening for tight-binding drug candidates in solutions. This technique demands only small amounts of samples, involves no radiolabeled materials and chemically immobilized ligands, and does not require changes in spectroscopic characteristics upon binding.

Most of biological events are triggered by receptor-ligand interactions such as protein-protein recognition involved in signal transduction pathways and protein-carbohydrate binding interactions used by the influenza virus to infect host cells. The determination of equilibrium binding constants, binding stoichiometries, and

kinetic rate oinstants would greatly contribute to the understanding of mechanisms and specificities of receptor proteins. The experimental methods involve a measured response at various ligand concentrations and fixed concentration of the receptor protein. The measured response can be related to the relative concentrations of free and bound ligand and, subsequently, to the binding constant; each of the different experimental methods can be related mathematically.

This method entails the covalent coupling of the receptor protein onto the surface of micro-beads containing a scintillator. Radio-labeled ligands that become bound to the immobilized receptor are sufficiently close to the fluor-containing bead to cause light emission, while the energy of free ligand is absorbed by the solution medium, resulting in no light generated. The stoichiometry of binding, the affinity of the binding sites for the ligand and the kinetics of association and dissociation. Using ACE for the screening of combinatorial libraries in solution will also along with a brief perspective on the future of ACE (Fig.1.4).

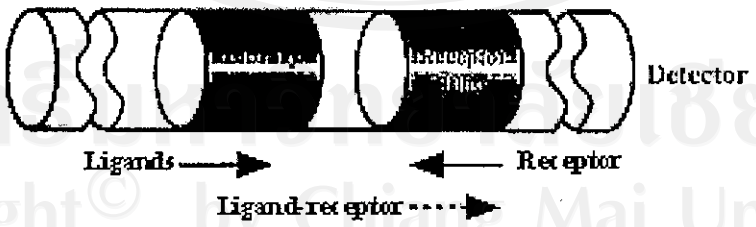


Figure 1.4 The biological events are triggered by receptor-ligand interactions. The different detectors can be used UV-VIS, fluorescence polarization, NMR, Mass and SPR.

1.1.3.3) CE with immobilized cells for drugs screening [70]

Many biological events are triggered by ligand/receptor interactions. For example, endothelin-1 (ET-1), a 21-peptide ligand locally produced in various cell types under different physiological stimuli, has a strong affinity toward endothelin receptor A (ET_A) located on the surface of a endothelial cell membrane [71-73]. This ligand/receptor interaction is coupled with G-protein, which triggers a series of biological events to induce an increase of intracellular calcium concentration, $[Ca^{2+}]_i$ [74]. Antagonism of the endothelin vasoconstrictor is a potential approach to the treatment of a variety of human diseases including hypertension and congestive heart failure [75]. Screening of natural and synthetic compounds based on the concept of ligand/receptor recognition is an indispensable strategy to search for endothelin receptor antagonists [76-77].

Affinity capillary electrophoresis (ACE) is a powerful separation method because it has the advantages of high resolution, small sample requirement, rapid sample throughput, and compatibility to biological conditions [78-82]. The components in an analyte can be separated by ACE due to their different electrophoresis motilities. By coupling with various sensitive detectors, the ACE technique is widely used to separate bioactive compounds and to determine the biomolecular non-covalent interactions [83-86]. When ACE is applied to screen active ligands in an analyte solution, the target receptor is often immobilized as the stationary phase on the inner wall of a capillary column [87-92]. However, this approach may encounter a problem in isolating of the desired receptors in sufficient quantity. Also, many membrane-bound receptors are unstable in isolation, as is the case of ET_A. Another problem is that receptors may lose their active conformations upon conjugation to capillary columns. We therefore

investigated the possibility of using whole cells with over-expression receptors, in lieu of the isolated receptors, as the stationary phase in ACE for the evaluation of active ligands [93,94].

In this thesis, the peptide and non-peptide ET_A antagonists (Fig.1.5) were satisfactorily resolved on ACE, in accordance to the order of their affinity and antagonist potency toward ET_A. We demonstrate that a whole cell stationary phase consist of ET_A-over-expressing CHO cells provides a successful ACE protocol for the screening of the ET_A-specific ligands.

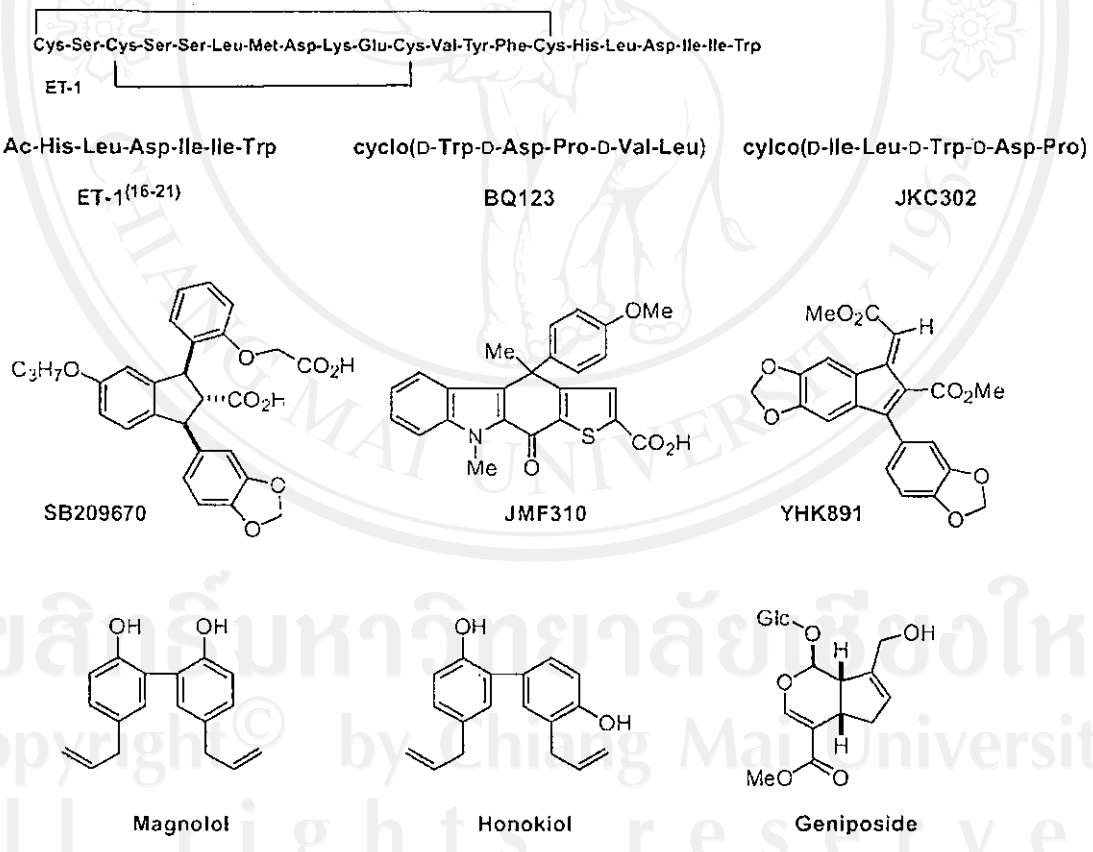


Figure 1.5 The examined substrates: peptides: ET-1(16-21), BQ123, JKC302 and ET-1 and non-peptide compounds: SB209670, JMF310, YHK891 and active herbal components Magnolol, Honokiol and Geniposide in this study.

1.2) Synthesis

1.2.1) Solid phase peptide synthesis (SPPS)[95]

The discovery of new peptides during the past decade has been phenomenal. New hormones, releasing factors, inhibitors, growth factors, toxins, ion carriers and antibiotics have been found, and the sequences of many new peptides and proteins of unknown function have been deduced from the nucleic acid studies. It is important to be able to make the parent compound to confirm its structure and in some cases to provide the peptide in larger quantities than can be conveniently obtained from natural sources. Peptides have become an increasingly important class of molecules in biochemistry, medicinal chemistry and physiology. Many naturally occurring, physiologically relevant peptides function as hormones, neurotransmitters, cytokines and growth factors. Peptide analogs that process agonist or antagonist activity are useful as tools to characterize their receptors, and to study their biosynthesis, metabolism and degradation. Peptide substrates of protease kinases and phosphatases are also used in enzyme kinetics, mechanism of action of enzymes and in the design of inhibitor.

Methods for synthesizing peptides are divided conveniently into two categories: solution or classical synthesis, the method has which evolved since the beginning of the twentieth century and retains value in large-scale manufacturing and for specialized laboratory applications and solid phase synthesis, which retains chemistry proved in solution, but adding a covalent attachment step that links the nascent peptide chain to an insoluble polymeric support

The solid phase peptide synthesis (SPPS) was conceived and developed for the purpose of providing a rapid, simplified and effective way to prepare peptides and small proteins by Bruce Merrifield and the optimization of supports, protecting groups

and coupling and deprotecting chemistries by a large number of researchers, it has become possible to obtain useful amount of peptides on a routine basis.

SPPS was introduced by Bruce Merrifield in 1963 in an effort to overcome many of the problems of peptide synthesis in solution as it was practiced at that time. SPPS was the first practical application of the use of insoluble, polymer-bound reagents in organic synthesis. Of the several possible approaches for polymer-assisted peptide synthesis, Merrifield chose to attach the C-terminal residue of the peptide to be synthesized to an insoluble polymer and grow the peptide chain toward the amino end of the peptide.

The fundamental premise of solid phase synthesis is that amino acids can be assembled into a peptide of any desired sequence while one end of the chain is anchored to an insoluble support. In practical SPPS the carboxyl terminus of the peptides is linked to the polymer. After the desired sequence of amino acids has been linked together on the support, a reagent can be applied to cleave the chain from the support and liberate the finished peptide into solution. All the reactions involved in the synthesis should be carried 100% to completion, so that a homogeneous product could be obtained. The great advantage of using a polymer-supported peptide chain is that all the laborious purification at intermediate steps in the synthesis is eliminated, and simple washing and filtration of the peptide-resin is substituted.

This basic idea of SPPS is illustrated in Fig.1.6. The insoluble support is a synthetic polymer which bears reactive groups (X). The amino acid which will form the C-terminal residue of the peptide to be synthesized is converted to a derivative in which its amino group is protected by a labile protecting group (L). Using appropriate chemistry, this derivative of the C-terminal amino acid is coupled to the reactive

polymer. At this point the repetitive cyclic part of SPPS begins. A reagent is applied to the protected aminoacyl polymer to remove the labile blocking group(L) from the amino acid residue. This reagent must not harm the link of the C-terminal residue to the polymer in any way.

If the amino acid attached to the polymer contains a side-chain reactive functional group, that functional group must be blocked by a stable blocking group (S) which will remain completely intact throughout the synthesis, but can be removed finally to yield the free peptide. Following removal of the labile protecting group, the next amino acid is coupled to the amino polymer by use of a suitable coupling reaction. Again, the α-amino group must be protected with the labile protecting group. This cycle of deprotection and coupling is then repeated with each amino acid which is to be incorporated into the peptide chain. Finally, after the entire blocked peptide has been assembled on the polymer support, a different type of reagent is applied to cleave the peptide from the polymer and allow it to be dissolved. The blocking groups which have protected side-chain functional groups must also be removed, and usually are chosen so that they can be removed simultaneously with cleavage of the peptide from the resin.

1.2.2) Nonpeptide compounds synthesis

Human endothelin-1(ET-1) is a 21 amino acid peptide that exhibits a potent vasoconstrictor activity, conceivably through its selective interaction with specific receptor subtypes (ET_A, ET_B and ET_C). ET-1 contains six highly conserved amino acid residues (His^{16}-Trp^{21}) at the C-terminus, and this hydrophobic C-terminal hexapeptide alone shows some affinity for ET_A receptor. Several antagonists including BQ-123 [cyclo (L-Lys-D-Val-L-Pro-D-Asp-D-Trp)][96] are designed on the basis of this peptide

structures that incorporate indole moieties. Some non-peptide endothelin antagonists also consist of indole scaffolds such as the indole-2-carboxylic acids PD159433[97] and SB209598[98] (Fig.1.7).

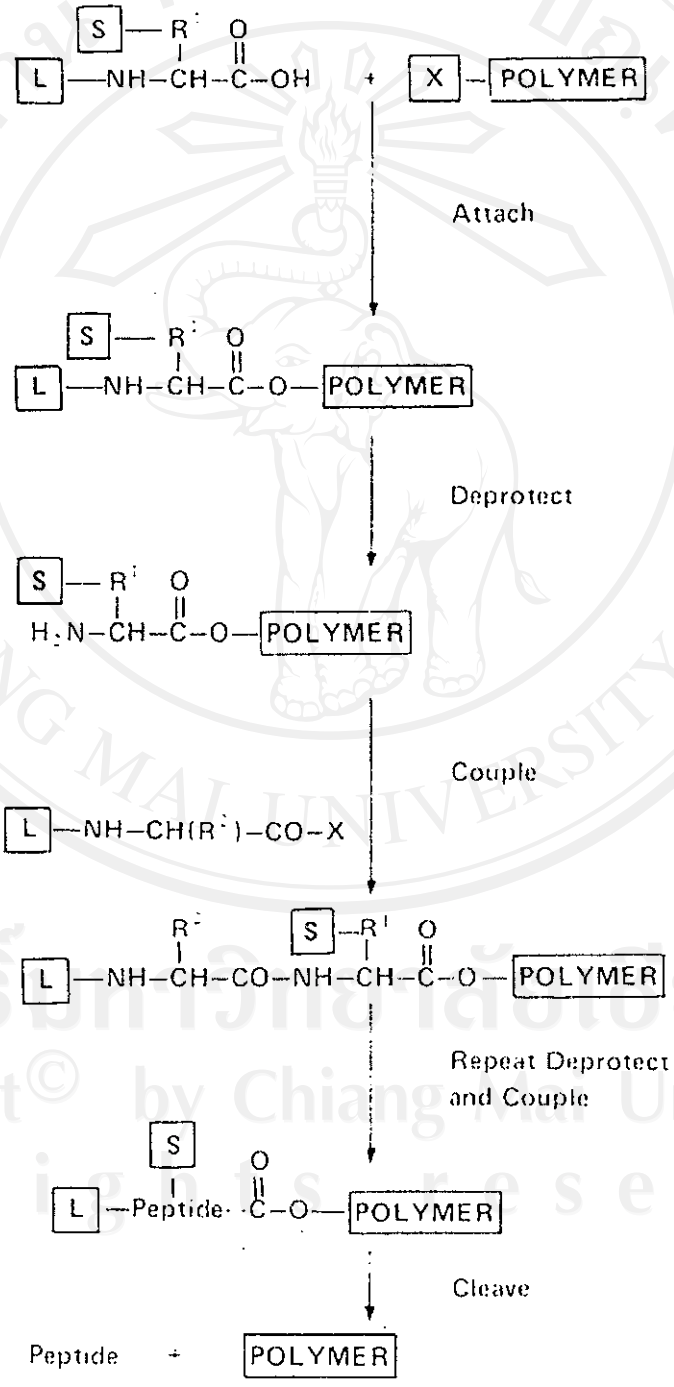


Figure 1.6 The scheme of solid phase peptide synthesis.

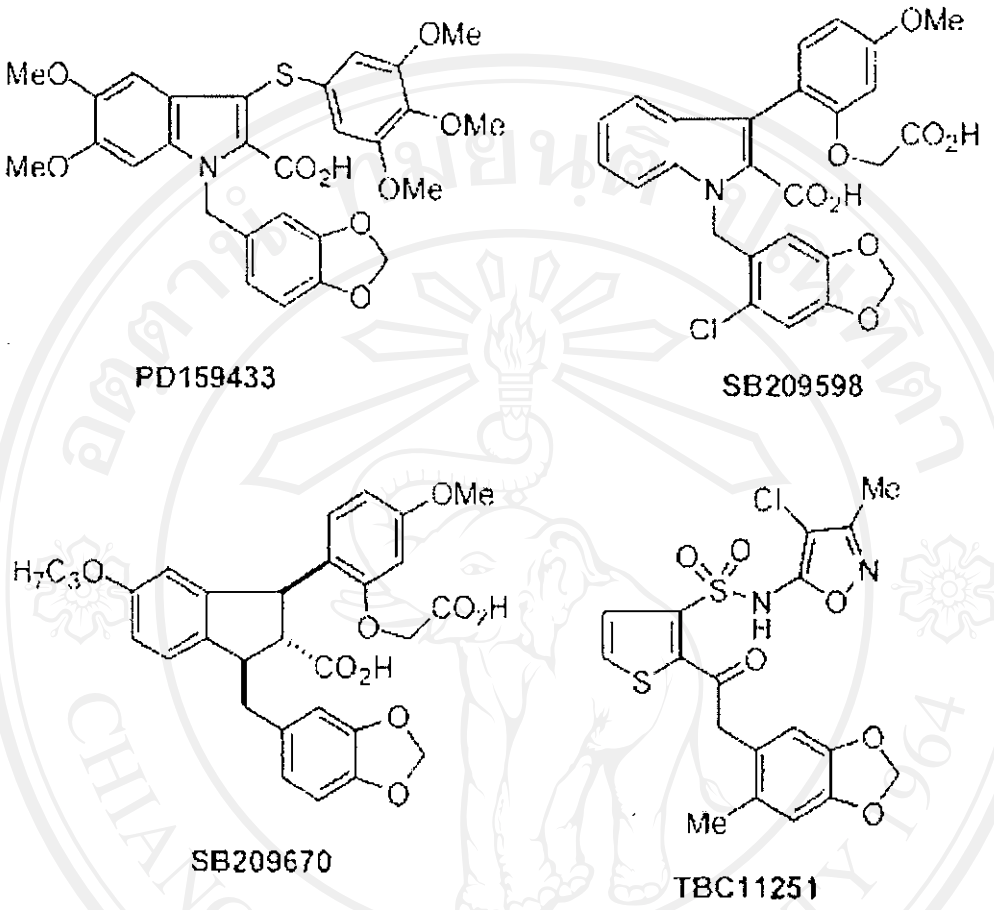


Figure 1.7 Some representative endothelin receptor antagonists constructed by the indole, indan and thiophene scaffolds.

1.2.2.1) Nonpeptide (I):Carbazolothiophene-2-carboxylic acid derivatives[98]

On the other hand, the molecular modeling indicates that an indan derivative SB209670[99] possesses two phenyl substituents to mimic the amino acid residues of Try-13 and Phe-14 in ET-1. The two carboxylic groups in SB209670 also mimic the Asp-18 residue and the *C*-terminus of ET-1, which ligate Zn^{2+} ion on binding with endothelin receptor. We speculated that a new class of carbazolothiophene derivatives bearing appropriate substituents might serve as endothelin receptor antagonists. Indeed,

5-benzyloxy-3-isopropoxy-benzothiophene-2-carboxylic acid[97] has been utilized as a lead compound for development of endothelin antagonists. A thiophene-3-sulfonamide TBC11251[100] is also known as an ET_A-selective antagonist, in which the sulfonamide moiety is considered as an isosteric of carbomate atructure carboxylic acid. We are thus interested in applying our established method of three-component coupling reactions of thiophene-2-carboxylate[101] to synthesize carbazolothiophene-2-carboxylate derivatives (Fig. 1.8) examined their antagonism against the binding of ET-1 with ET_A receptor.

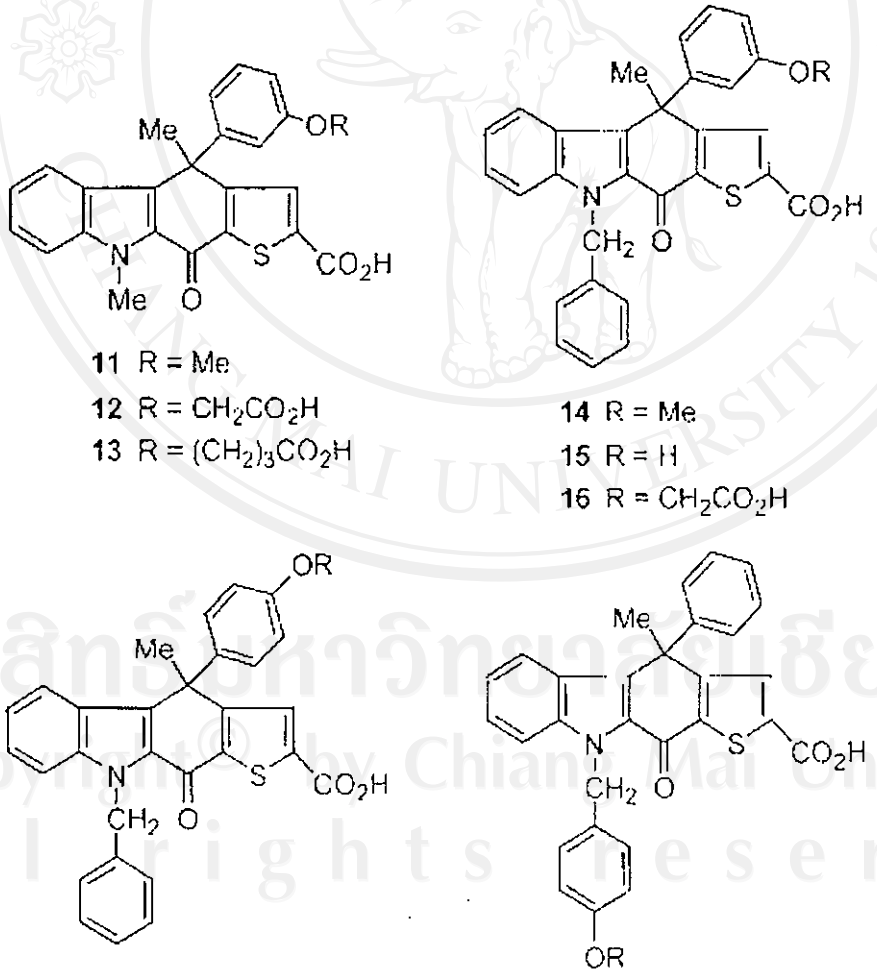
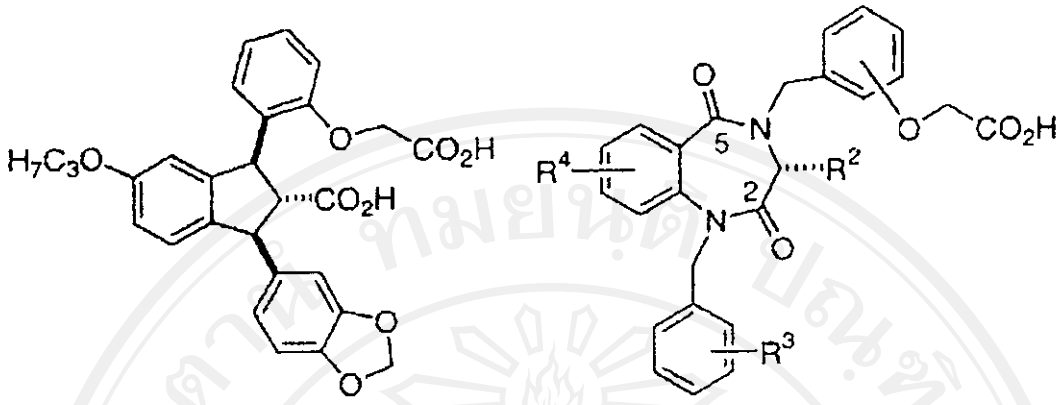


Figure 1.8 Carbazolothiophene-2-carboxylate derivatives.

1.2.2.2) Nonpeptide (II):1,4-Benzodiazepine-2,5-dione derivatives

Among numerous endothelin receptor antagonists, an indan derivative SB209670 possesses two phenyl substituents to mimic the amino acid residues of Tyr-13 and Phe-14 in ET-1. The two carboxylic groups in SB209670 also mimic the Asp-18 residue and the *C* terminus of ET-1, which ligates the Zn^{2+} ion on binding with endothelin receptor. In the process of searching for the nonpeptide endothelin antagonists, we speculated that 1,4-benzodiazepine-2,5-dione derivatives bearing appropriate substituents might serve for this purpose[102]. The benzodiazepine core provides a nearly planar platform as that of the indan ring in SB209670 (Fig. 1.9). A library of 1,4-benzodiazepine-2,5-dione dicarboxylate derivatives containing aryl substituents at N_1- and N_4-positions to mimic the amino acid residues of Tyr-13, Phe-14, and Asp-18 in endothelin-1 is established by using the starting materials of R-amino esters, hydroxybenzaldehydes, nitrobenzoyl chlorides, and benzyl bromides in a polyethylene resin-bound liquid-phase synthesis[103]. For a better binding affinity with the endothelin receptors, carboxylic groups and other substituents may be introduced to various sites of the benzodiazepine scaffold. 1,4-Benzodiazepine-2,5-dione and its analogues represent an important class of bioactive molecules. These compounds show remarkable potency in various biological targets, including antithrombotics, antibiotics, and antitumor activities. Many efforts have been exerted on the synthesis of this class of bioactive compounds[104].



SB299670 1,4-Benzodiazepine-2,5-dione derivatives

Figure 1.9 1,4-Benzodiazepine-2,5-dione derivatives bearing carboxylic groups are designed to mimic the endothelin receptor antagonist SB209670, which contains two aryl substituents flanking the nearly; lanar core of indan ring.

1.3) Biological activity assay: Measurement of intracellular calcium concentration($[Ca^{2+}]_i$ assay)

The relaxation or contraction of the artery and vein has significant relationship with the endothelial cell[105,106] (Fig. 1.10) .

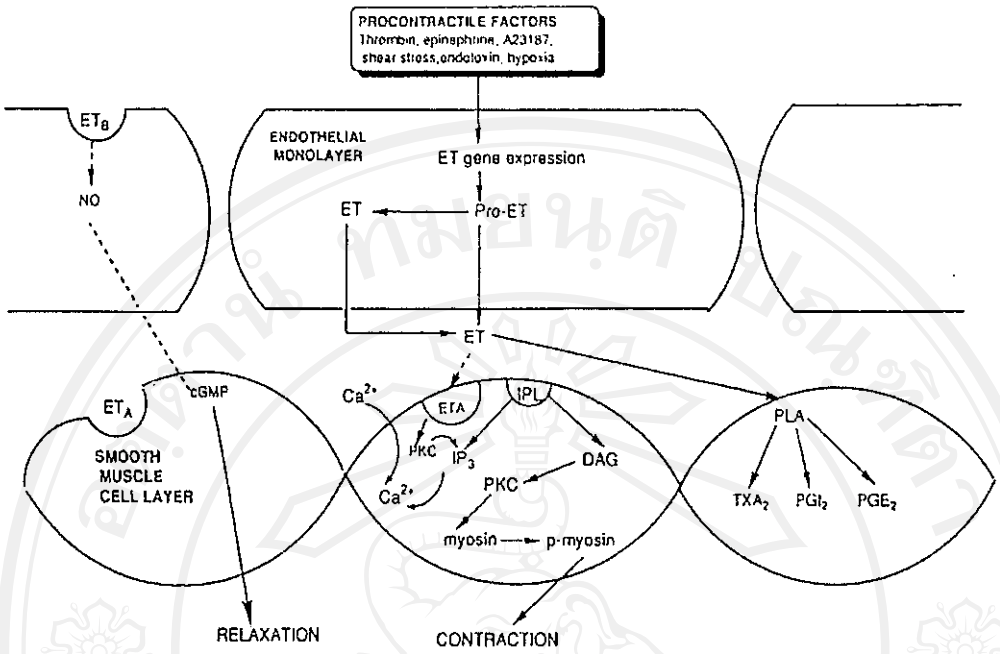


Figure 1.10 Intracellular signaling.

The relaxation of the blood vessel is derived from the action of many kinds of vasodilators excreted from the endothelial cell. The endothelium-derived relaxing factor (EDRF) is the most important one, which is triggered from the catalysis of nitric oxide synthase (NOS) to L-arginine, releasing nitric oxide (NO). Nitric oxide stimulates and activates soluble guanylate cyclase, resulting to the accumulation of cyclic guanosine monophosphate(c-GMP), then inducing the relaxation of the smooth muscle. Besides the releasing of EDRF, endothelium-derived contracting factors (EDCF) are excreted simultaneously. The constituents of EDCF are superoxide anions, endoperoxide, thromboxane A_2 (TxA_2) and ET_S. The normal endothelial cells excrete more EDRF than EDCF, but when this phenomenon is deviated, the symptom of high blood pressure is shown in Fig. 1.11.

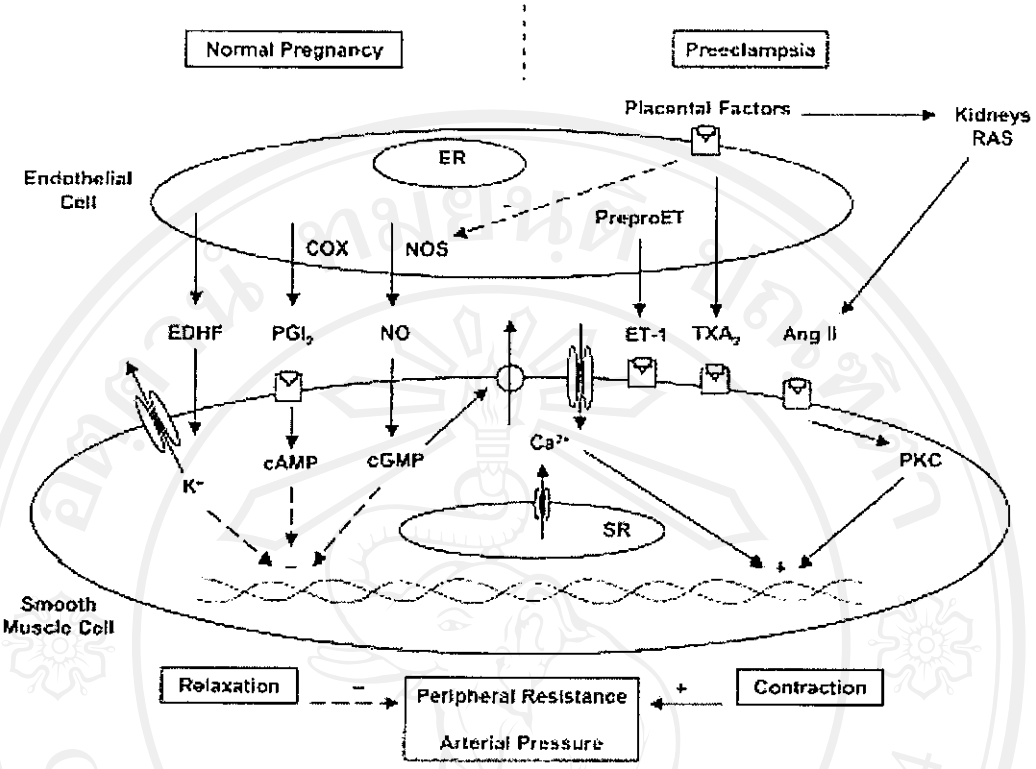


Figure 1.11 The mediation of endothelin to the contraction of blood vessel via the consequent intracellular messenger.

The binding of ET-1 to ET_A can activate phospholipase C (PLC) and phospholipase A_2 (PLA_2), also stimulate voltage-dependent calcium channel (VDC) and receptor operated calcium channel (ROC). PLC can catalyze the production of inositol trisphosphate (IP_3) and diacylglycerol (DG). DG activates protein kinase C (PKC), then the result indirectly induces the smooth muscle contraction. The other factors modulating the smooth muscle contraction are prostacyclin (PGI_2), prostaglandin E_2 (PGE_2), TXA_2 and all of them are derived from PLA_2. IP_3 can induce the release of calcium ion, Ca^{2+}, from its storage, and the stimulation of VDC and ROC also increase intracellular free Ca^{2+}, then both increases of free Ca^{2+} may stimulate the smooth muscle contraction of blood vessel[107,108].

The concentration of Ca^{2+} is one kind of intracellular second messengers, and its increasing concentration may induce the secretion, the activation of enzyme, besides the contraction. The intracellular concentration of Ca^{2+} is around the range of nM, but its extracellular concentration is higher up to 2 mM. Based on this situation, once the cell accepts the extracellular message, the intracellular concentration of Ca^{2+} is increased[109]. The source of increased Ca^{2+} is derived at least from two kinds of pathways. One is the entrance of extracellular calcium via the calcium channels on the cell membrane, and this type of channel is classified into four groups: (1) voltage-operated calcium channel regulated by the menbrane voltage[110], (2) receptor-operated calcium channel, and the receptor itself being the calcium channel, the receptor of ATP[111], (3) second messenger-operated calcium channel and its function is contributed to the IP_3 and camp responses[112], (4) store-dependent calcium channel, and it means that the depletion of intracellular calcium stores activates a calcium current into the cell[113]. The other pathway is derived from the intracellular calcium pools, which is also divided into four: (1) IP_3-sensitive calcium pool, and only the binding of IP_3 and its receptor, located on the membrane of this pool, opens the storage of Ca^{2+} [114], (2) GTP-sensitive calcium pool, and the releasing of Ca^{2+} is due to the existence of GTP[115], (3) calcium or caffeine induced calcium release, (4) cyclic ADP ribose sensitive calcium pool.

To evaluate the efficiency of endothelin receptor antagonists, we measured the ET-1-induced increase of intracellular free $[Ca^{2+}]_i$ in intact cells by using Fura-2 as a ratiometric Ca^{2+} indicator. This compound combines an 8-coordinate tetracarboxylate chelating site with stilbene chromophores, then the calcium-free or calcium-bound Fura-2 may exhibit quite strong fluorescence. Its binding of Ca^{2+} shifts the fluorescence

excitation spectrum to shorter wavelength. Owing to the better selectivity for Ca^{2+} over other divalent cations, particularity of its wavelength sensitivity to Ca^{2+}, the dye, Fura-2 make itself as the preferred fluorescent indicator for many intracellular applications, especially in single cells, adherent cell layers, or bulk tissues.

From the ratio of absorption of the fluorescence excitation, the concentration of intracellular calcium can be deduced, which was based on the method offered by Grynkiewicz[124] : The purpose of the addition digitonin is to make the complete binding of Fura-2 with Ca^{2+}, yielding the value of R_{max}; oppositely, the subsequent addition of EGTA discards Ca^{2+} completely, yielding the value of R_{min}. The concentration or intracellular calcium is figured out by the following equation:

$[Ca^{2+}]=K_d \times (sf / sb) \times [(R-R_{min}) / (R_{max}-R)]$

K_d : the dissociation constant of Fura-2 to Ca^{2+}

R : the ratio of fluorescence intensity at 340 nm to 380 nm

R_{min} : the ratio of fluorescence intensity at 340 nm to 380 nm when the concentration of Ca^{2+} was close to zero

R_{max} : the ratio of fluorescence intensity at 340 nm to 380 nm when the concentration of Ca^{2+} was close to saturation

sf / sb : the ratio of fluorescence intensity at 380 nm when the concentration of Ca^{2+} was close to zero (sf) and when the concentration of Ca^{2+} was close to saturation (sb)

The evaluated concentration of eleven antagonists was 10^{-6} M, then each of them was treated with 10^{-7} M of ET-1.

1.4) Literature reviews

Many biological events are triggered by ligand/receptor interactions. For example, endothelin-1 (ET-1), a 21-peptide ligand locally produced in various cell types under different physiological stimuli, has a strong affinity toward endothelin receptor A (ET_A) located on the surface of a endothelial cell membrane. This ligand/receptor interaction is coupled with G-protein, which triggers a series of biological events to induce an increase of intracellular calcium concentration, $[Ca^{2+}]_i$. Antagonism of the endothelin vasoconstrictor is a potential approach to the treatment of a variety of human diseases including hypertension and congestive heart failure. Screening of natural and synthetic compounds based on the concept of ligand/receptor recognition is an indispensable strategy to search for endothelin receptor antagonists.

Affinity capillary electrophoresis (ACE) is a powerful separation method because it has the advantages of high resolution, small sample requirement, rapid sample throughput, and compatibility to biological conditions. The components in an analyte can be separated by ACE due to their different electrophoresis motilities. By coupling with various sensitive detectors, the ACE technique is widely used to separate bioactive compounds and to determine the biomolecular non-covalent interactions. When ACE is applied to screen active ligands in an analyte solution, the target receptor is often immobilized as the stationary phase on the inner wall of a capillary column. However, this approach may encounter a problem in isolating of the desired receptors in sufficient quantity. Also, many membrane-bound receptors are unstable in isolation, as is the case of ET_A. Another problem is that receptors may lose their active conformations upon conjugation to capillary columns. We therefore investigated the

possibility of using whole cells with over-expression receptors, in lieu of the isolated receptors, as the stationary phase in ACE for the evaluation of active ligands.

Human endothelin-1 (ET-1) is a 21 amino acid peptide that exhibits a potent vasoconstrictor activity, conceivably through its selective interaction with specific receptor subtypes (ET_A, ET_B and ET_C). ET-1 contains six highly conserved amino acid residues (His-16, Trp-21) at the *C*-terminus, and this hydrophobic *C*-terminal hexapeptide alone shows some affinity for ET_A receptor. Several antagonists including BQ123 [cyclo(L-Leu-D-Val-L-Pro-D-Asp-D-Trp)] are designed on the basis of this peptide structures that incorporate indole moieties. Some non-peptide endothelin antagonists also consist of indole scaffolds such as the indole-2-carboxylic acids PD159433 and SB209598. On the other hand, the molecular modeling indicates that an indan derivative SB209670 possesses two phenyl substituents to mimic the amino acid residues of Tyr-13 and Phe-14 in ET-1. The two carboxylic groups in SB209670 also mimic the Asp-18 residue and the *C*-terminus of ET-1, which ligate Zn^{2+} ion on binding with endothelin receptor. In the process of searching for the nonpeptide endothelin antagonists, we speculated that two new classes of 1,4-benzodiazepine-2,5-dione dicarboxylate derivatives and carbazolothiophene-2-carboxylic acid derivatives bearing appropriate substituents might serve as endothelin receptor antagonists.

The 1,4-benzodiazepine-2,5-dione derivatives bearing appropriate substituents might serve for this purpose. The benzodiazepine core provides a nearly planar platform as that of the indan ring in SB209670. The N1- and N4-positions may be implanted with the desired aryl substituents. For a better binding affinity with the endothelin receptors, carboxylic groups and other substituents may be introduced to various sites of the benzodiazepine scaffold. 1,4-Benzodiazepine-2,5-dione and its

analogues represent an important class of bioactive molecules.4 These compounds show remarkable potency in various biological targets, including antithrombotics, antibiotics, and antitumor activi-ties. Many efforts have been exerted on the synthesis of this class of bioactive compounds.

The carbazolothiophene-2-carboxylic acid derivatives bearing appropriate substituents might serve for this purpose. A thiophene-3-sulfonamide TBC11251 is also known as an ET_A-selective antagonist, in which the sulfonamide moiety is considered an isosteric of carbomate structure . We are thus interested in applying our established method of three-component coupling reactions of thiophene-2-carboxylate to synthesize carbazolothiophene-2-carboxylate derivatives, and examined their antagonism against the binding of ET-1 with ET_A receptor.

In this study, we demonstrate that a whole cell stationary phase consisting of ET_A-over-expressing CHO cells provides a successful ACE protocol for the screening of the ET_A-specific ligands. The peptide and non-peptide ET_A antagonists were satisfactorily resolved on ACE, in accordance to the order of their affinity and antagonist potency toward ET_A. A series of nonpeptide compounds bearing the indole and thiophene rings were prepared in an expedient fashion. The functional assay indicated that one of these samples can serve as a lead compound for future exploration of potent endothelin receptor antagonists. The structure-activity relationship also awaits further investigation.

1.5) Purpose of the study

The goal of this proposal is to develop a practical capillary electrophoresis method which can pick out biologically active compounds from combinatory library

using receptor on the cell membrane as stationary phase. Endothelin receptor that expresses on Chinese hamster ovary (CHO) cell was chosen as stationary phase to pack in the empty capillary column to separate antagonists. Although various endothelin 1 (ET-1) analogues have been shown to have some antagonistic activity, none of the compounds so far considered, have sufficiently high receptor specificity and affinity. Elliott showed that SB209670 and ET-1 possess several common spatial arrangements. The two hydrophobic groups of SB209670 have similar spatial relationship to the side chains of Tyr-13, Phe-14 and Trp-21 of ET-1. The carboxyl group of SB209670 is relative to the spatial arrangement of Asp-18 or C-terminal carboxyl group of ET-1.