The analysis of the anti-inflammatory mechanism of the novel stroke drug SMTP

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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIS</td>
<td>acute ischemic stroke</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole</td>
</tr>
<tr>
<td>AP</td>
<td>anti-plasmin</td>
</tr>
<tr>
<td>ASA</td>
<td>aminosalicylic acid</td>
</tr>
<tr>
<td>AUDA</td>
<td>12-((3-adamantan-1-yl-ureido)dodecanoic acid</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>Cterm-EH</td>
<td>C-terminal epoxide hydrolase</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DALYs</td>
<td>disability-adjusted life-years</td>
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<tr>
<td>DHET</td>
<td>dihydroxyeicosatrienoic acid</td>
</tr>
<tr>
<td>EET</td>
<td>epoxyeicosatrienoic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>ETE</td>
<td>oxo-eicosatetraenoic acid</td>
</tr>
<tr>
<td>HETE</td>
<td>hydroxyeicosatetraenoic</td>
</tr>
<tr>
<td>HPETE</td>
<td>hydroperoxyeicosatetraenoic</td>
</tr>
<tr>
<td>ICAM</td>
<td>intracellular adhesion molecule</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitrogen oxide synthase</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LOX</td>
<td>lipooxygenase</td>
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<tr>
<td>LT</td>
<td>leukotriene</td>
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<tr>
<td>LX</td>
<td>lipoxin</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOX</td>
<td>nicotinamide adenine dinucleotide phosphate oxidase</td>
</tr>
<tr>
<td>Nterm-phos</td>
<td>N-terminal phosphatase</td>
</tr>
<tr>
<td>ODS</td>
<td>octadecylsilane</td>
</tr>
<tr>
<td>PAI</td>
<td>plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PAN</td>
<td>plasminogen/apple/nematode</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PHOME</td>
<td>3-phenyl-oxiranyl)-acetic acid</td>
</tr>
<tr>
<td>cyano-(6-methoxy-naphthalen-2-yl)-methyl ester</td>
<td></td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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</table>
sct-PA  single-chain tissue-type plasminogen activator
scu-PA  single-chain urokinase-type plasminogen activator
SD     standard deviation
SDS    sodium dodecylsulfate
sEH    soluble epoxide hydrolase
SMTP   Stachybotrys microspora triprenyl phenol
TAFI    thrombin activated fibrinolysis inhibitor
tct-PA  two-chain tissue-type plasminogen activator
tcu-PA  two-chain urokinase-type plasminogen activator
TNBS   2,4,6-trinitrobenzenesulfonic acid
TNF    tumor necrosis factor
t-PA    tissue-type plasminogen activator
TX      thromboxane
u-PA    urokinase-type plasminogen activator
VCAM   vascular cell adhesion molecule
VEGF   vascular endothelial growth factor
α2-M   α2- macroglobulin
Abstract

Stroke is a major cause of death and disability worldwide. Currently, only a thrombolytic therapy using tissue plasminogen activator (t-PA) is approved to treat acute ischemic stroke, however, it is used only in 2–9% of the patients, due to its narrow therapeutic window and the risk of intracranial hemorrhage. Thus, an alternative strategy is needed to rescue patients who cannot receive t-PA-based therapy.

SMTPs are a family of fungal metabolites isolated from *Stachybotrys microspora*. The SMTP molecule consists of a core unit (tricyclic $\gamma$-lactam with a geranylmethyl side-chain) and varying $N$-linked side-chain. Some of the SMTP congeners have “plasminogen modulation” activity, which is the enhancement of plasminogen activator-catalyzed activation of plasminogen, zymogen of plasmin that plays an essential role in clot degradation in the blood vessel. SMTP-7, one of the most potent congeners, effectively treats ischemic stroke in several animal models, some of which are too severe to be treated by t-PA. It has been postulated that an unexpected anti-inflammatory action is involved in the excellent therapeutic effect. Based on these properties, one of the congeners of SMTPs is under clinical development as a novel therapeutic agent for treatment of ischemic stroke. In this thesis, I describe the analysis of the anti-inflammatory mechanism of SMTPs.

First, we identified the anti-inflammatory target of SMTP-7. We found that SMTP-44D, a congener of SMTPs without a plasminogen modulation activity, as well as SMTP-7, was effective in treating some animal models of inflammatory diseases (Guillain-Barré syndrome, ulcerative colitis, and Crohn’s disease), which were irrelevant to thromboembolic complications. The results demonstrated that the anti-inflammatory action of SMTPs was independent of the plasminogen modulation activity, suggesting the presence of an alternative target for anti-inflammation. We prepared an affinity matrix that had a core unit of SMTP linked via an $N$-linked side-chain. This enabled the purification of soluble epoxide hydrolase (sEH). sEH is a bifunctional enzyme composed of the C-terminal epoxide hydrolase domain (Cterm-EH) and N-terminal lipid hydrolase domain (Nterm-phos). Cterm-EH degrades anti-inflammatory epoxy-fatty acids into inactive dihydroxy-fatty acids. Inhibition or genetic deficiency of sEH results in suppression of inflammation. SMTP-7 and SMTP-44D inhibited both Cterm-EH and Nterm-phos *in vitro*. Detailed kinetic analyses using SMTP-0, the simplest SMTP congeners, revealed that the inhibitions of Cterm-EH and Nterm-phos were competitive and pseudononcompetitive, respectively. These results suggest that SMTP-0 binds to two distinct sites in sEH. SMTP-7 inhibited the hydrolysis of epoxyeicosatrienoic acid in cultured HepG2 cells and reduced plasma level of dihydroxyeicosatrienoic acid, the
hydrolysis product of epoxyeicosatrienoic acid in a rat model of Guillain-Barré syndrome. Thus, I propose that sEH is responsible for the anti-inflammatory action of SMTP.

SMTP-0, SMTP-7, and SMTP-44D had distinct selectivity toward plasminogen, Cterm-EH, and Nterm-phos. Thus, SMTPs differently affect these three targets depending on the structure of the N-linked side chain. Congeners selective to both plasminogen and Cterm-EH are promising candidates for a stroke drug, whereas congeners specific to Cterm-EH or Nterm-phos may be useful in other indications, including inflammatory diseases. In addition, a specific inhibitor of Nterm-phos can be a tool for investigations of its physiological role, which still remains elusive. Thus, we next examined 39 SMTP congeners with various N-linked side-chains for their activity toward plasminogen activation, Cterm-EH, and Nterm-phos to elucidate structure-activity relationships among the three targets.

Many SMTP congeners, including SMTP-0 that lacks N-linked side chain, were inhibitory to both Cterm-EH and Nterm-phos, indicating the essential role of the core unit of SMTPs in these activities. SMTP-7, SMTP-19, SMTP-25, and SMTP-28, congeners with an aromatic N-linked side-chain with a carboxyl group, showed excellent activity toward both plasminogen and Cterm-EH. Other congeners with an N-phenylcarboxylic acid showed significant dual activity. Currently, SMTP-7 is not orally available. This may be partly due to its large N-linked side-chain. Congeners with a smaller N-linked structure may help development of an orally available SMTP. Congeners with a hydrophobic N-linked side-chain were specific for inhibition of Nterm-phos rather than Cterm-EH. These congeners can be used to study physiological role for Nterm-phos.

In conclusion, we have revealed the anti-inflammatory mechanism of SMTP-7 as that it inhibits sEH. The sEH inhibition results in anti-inflammatory responses through a change in the level of epoxy-fatty acids. The selectivity of SMTPs toward plasminogen, Cterm-EH, and Nterm-phos can be altered by the modification of the N-linked side-chain. These findings provide a means to design ideal congeners by modifying the N-linked side-chain with respect to favorable bioactivity and bioavailability.
Chapter 1 Introduction to SMTPs, a Novel Therapeutic for Ischemic Stroke

1.1 Ischemic stroke

1.1.1 General aspects

Stroke is the second leading cause of death worldwide, killing more than 6 million people every year (1,2), and is also estimated as the sixth leading cause of disability-adjusted life-years (DALYs): the sum of life-years lost as a result of premature death and years lived with disability adjusted for severity (3). Obesity, hypertension, diabetes mellitus, dyslipidemia, alcohol intake, smoking, reduced physical activity, and psychosocial stress and depression are known as the risk factors for stroke which may come from one's lifestyle (4). As urbanization increases these risks, the number of stroke occurring in the next decade is predicted to increase not only in advanced countries but also in developing countries (5). Improving the case-fatality rates and long-term disability after stroke are imperative needs.

1.1.2 Pathological relevance

More than 70% of acute stroke incidents are classified as acute ischemic stroke (AIS) (6). AIS occurs when a cerebral artery is occluded and blood flow in an artery to the brain is blocked. The brain cells and tissues begin to die within minutes due to the lacks of oxygen and glucose. AIS is usually caused by either an atherothrombosis of a cerebral artery, or an embolus (clot or plaque debris) traveled from an extracerebral region (7). The blood flow interference initiates a series of harmful events in the central nervous system, known as the ischemic cascade. In the ischemic condition, anaerobic glycolysis accumulates lactic acid in the central nervous system, which raises cerebral acidosis and activates acid-sensitive ion channels for Na\(^{2+}\) Ca\(^{2+}\) effluxes to the cytosol (8). The neuronal cells are stimulated by the intracellular Ca\(^{2+}\) to secrete the excitatory neurotransmitter glutamic acid to the extracellular space. Secreted glutamate activates N-methyl-D-aspartate (NMDA) receptor (9,10) and α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) receptor ion channels (11), which ultimately accelerate influx of Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\) ions. The function of ion pumps to redistribute these ions is impaired because of the ATP starvation (12). The disturbed ion homeostasis causes mitochondrial dysfunction and activation of caspases, leading to the neuronal apoptosis and necrosis (13-15) (Fig. 1.1).

The blood reperfusion can also raises catastrophic events turning the penumbra (damaged but salvageable region surrounding a necrotic core) into the necrotic tissue, known as the reperfusion injury. It progresses even several hours to days after the reperfusion (16). The restored oxygen supply produces harmful reactive oxygen species (ROS) such as hydroxyl radical, superoxide anion radical, hydrogen peroxide, and nitrogen oxide. Several enzymes are
suggested to produce these ROS in the reperfused tissue, such as neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), xanthine oxidase, NADPH oxidase 4 (NOX4) (17,18). The oxidative stress upregulates the expression/secretion/activity of numerous kinds of tissue degenerative factors, such as the pro-inflammatory cytokines (TNF-α, IL-1β), adhesion molecules (ICAM-1, VCAM-1, E-selectin), factors permeabilizing the blood-brain-barrier (BBB) (VEGF, bradykinin), and proteases (MMP-2, MMP-9) (19-29). VEGF and bradykinin enhance cerebral vascular permeability, by inducing the endocytosis of VE-cadherin (a major adhesion molecule maintaining the tight-junction of BBB) on the endothelial cells (30), or by relaxing the vascular smooth muscle cells (24,31,32), respectively. MMPs degrade neurovascular units, which also permeabilize BBB (28,33). The pro-inflammatory cytokines themselves are cytotoxic (34), and they, together with the adhesion molecules, activate and recruit circulating leukocytes (macrophages, neutrophils, and T cells) to the infarct tissue (35). Microglia, the macrophage-like cells resident in a central nervous system, are also activated in response to reperfusion (36). These leukocytes have important roles in scavenging died cells and in secreting regenerative factors such as TGF-β, however, they also produces ROS, pro-inflammatory cytokines, bradykinin and MMPs (37). These positive feedbacks can aggravate tissue injury, and finally cause life-threatening edema and hemorrhagic transformation (26,38) (Fig. 1.1). Several associations are observed between the markers of reperfusion injury (such as plasma TNF-α and MMP-9) and a poor clinical outcome (39,40), thus the reperfusion injury is likely to contribute to the pathology of AIS not only in animal models, but also in humans.

**FIGURE 1.1. Biological process of tissue injury triggered by ischemia/reperfusion.**

### 1.1.3 Treatment

Early recanalization of the blood flow is necessary for a good outcome in AIS (41). In
this point of view, the thrombolytic therapy has been developed. Currently, recombinant tissue plasminogen activator (t-PA) is a only thrombolytic agent approved for the treatment of human AIS. Patients treated with intravenous t-PA administration were at least 30 % more likely to have minimal or no disability at 3 months (42,43), however, it can be used in a small fraction (1.8–8.9%) of patients (44,45), because it can induce severe hemorrhagic transformation in the infarct area (42,43,46). The poor outcome and the risk of the hemorrhage correlates with the delay of the administration (47). Cell based biological and biochemical studies show that t-PA upregulates pro-inflammatory pathways and the expression of tissue degenerative MMPs via transmembrane receptors (48-50). Induction of pro-inflammatory mediators and MMPs are also seen in vivo when the administration of t-PA is delayed (51). Therefore, the narrow therapeutic-window of t-PA is likely to be due to its deterioration of reperfusion injury by itself. The beneficial effect of t-PA is not predicted to outweigh its risk unless it is administered within 3 or 4.5 hours of the stroke onset (47). Currently, there is no way to treat AIS patients in a hyper-acute phase, who are outside the therapeutic-time window of t-PA.

The concept of neuroprotectant is an alternative strategy targeting reperfusion injury. Theoretically, neuroprotectants are effective in the AIS patients who's receiving a drug is delayed. As described above, numerous biological processes are involved in the reperfusion injury, therefore many bioactive compounds with various mechanisms have been developed as neuroprotectants (52-55), including free radical scavengers, Ca\(^{2+}\) channel blockers, glutamate receptor antagonists, anti-adhesion molecule antibodies, immunosuppressors, and anti-inflammatory agents. Although many of them successfully treated AIS in animal models, none of them showed therapeutic effect in the human patients. These results seem to be partly due to the discrepancies in the conditions of treatment in clinical and non-clinical trials (56). While numerous clinical studies for neuroprotectants resulted in failure, the potential of neuroprotectants to rescue patients who cannot receive t-PA-based therapy is attractive enough to continue development.

1.2 Fibrinolysis

1.2.1 Functional importance of coagulation/fibrinolysis

The fluidity of circulating blood is stabilized and modulated by coagulation and fibrinolysis. Coagulation is the process of clot formation, via series of cascade process: tissue factors activate prothrombin to thrombin, which cleaves fibrinogen into fibrin, and fibrin, together with erythrocytes and activated platelets, forms a clot (57,58). Fibrinolysis is the process of degrading clot-forming fibrin to the soluble fragments, which is mediated by the
Plasmin. Plasminogen is activated by plasminogen activators such as t-PA and urokinase plasminogen activator (u-PA) to form plasmin (Fig. 1.2). The balance between coagulation and fibrinolysis is quite important. Impaired coagulation cascade, such as the deficiency of factor VIII or factor IX, causes severe hemophilia (57), whereas the deficiency of plasminogen or its activators causes spontaneous/excess thrombosis, impaired thrombolysis, epithelial ulcers, and impaired wound healing (59,60).

![FIGURE 1.2. Pathways of coagulation and fibrinolysis.](image)

### 1.2.2 Regulation of plasmin activity

In the unusual physiological condition where plasminogen is constitutively activated, the resulting plasmin rapidly runs short circulating fibrinogen, resulting in hemorrhage within several minutes (61). Under normal physiological conditions, plasmin is present in blood (and extravascular fluids) as an inactive form, plasminogen (60). Its activity is cardinally regulated by activation and inhibition process.

$t$-PA and $u$-PA are endogenous serine protease activating plasminogen. $t$-PA is primarily expressed by endothelial cells as a single-chain form (sct-PA) (60). Both plasminogen/plasmin and $t$-PA have affinity to fibrin, and this co-localization enhances the catalytic efficacy of plasmin to cleave the peptide bond of sct-PA between Arg275-Ile276 into a disulfide-linked two-chain form $t$-PA (tct-PA). sct-PA and tct-PA cleave plasminogen into active plasmin (62).
u-PA is primarily expressed by renal epithelium as a single-chain form (scu-PA). The peptide bond of scu-PA between Lys158-Ile159 is cleaved by plasmin and another serine protease kallikrein into the disulfide-linked two-chain form (tcu-PA). This cleavage enhances the efficacy of u-PA to activate plasminogen by ~200 folds (63). While the plasminogen activation by u-PA is efficiently enhanced in the presence of fibrin, the binding affinity of u-PA to fibrin is shown to be much lower than that of t-PA. It is suggested that the influence of fibrin to the u-PA-mediated plasminogen activation is mainly due to the conformational change of plasminogen binding to fibrin. Free plasmin released from target protein is immediately neutralized by circulating inhibitors such as $\alpha_2$-antiplasmin ($\alpha_2$-AP), and $\alpha_2$-macroglobulin ($\alpha_2$-M), via the formation of the irreversible complexes. Owing to those process described above, plasmin is able to work only within the neighborhood of these the target proteins, preventing detrimental proteolysis.

In addition to these activators and inhibitors, several mechanisms is involved in activation of plasminogen. TAFI is a carboxypeptidase present in plasma, cleaving the carboxy-terminus of lysine residues of fibrin where plasminogen and t-PA interact with each other, thus fibrin mediated enhancement of plasminogen activation is suppressed (60). The activities of t-PA and u-PA are irreversibly inhibited by plasminogen activator inhibitor-1 (PAI-1) (60,64).

1.2.3 Molecular mechanisms of plasminogen activation

Full-length plasminogen (Glu-plasminogen) consists of 791 residues and seven domains, which comprises an N-terminal plasminogen/apple/nematode (PAN) domain, 5 kringle domains (KR1-5) and a serine protease domain (SP). Plasminogen adopts two distinct conformations, termed closed and open conformations. Glu-plasminogen circulates in a closed conformation that is not readily activated by t-PA or u-PA. Glu-plasminogen can afford an open conformation when bound to fibrin. The removal of the PAN domain (by the cleavage between Lys$_{76}$ and Lys$_{77}$) by plasmin during pre-activation produces an alternative zymogen form called Lys-plasminogen, which adopts an open conformation with elevated affinity to fibrin (65,66). Glu and Lys-plasminogen in the open conformation is thought to have a flexible ‘beads on a string’ formation. In the open conformation, the activation loop present between KR5 and SP is exposed, and then t-PA or u-PA cleaves the peptide bond between Arg$_{561}$ and Val$_{562}$ of the activation loop to convert plasminogen to plasmin. PAN and KRs play important roles in maintaining the closed conformation of plasminogen and in switching it to open conformation when plasminogen contacts fibrin. In closed plasminogen, the activation loop is occluded in KR4 and the liker region between KR3 and KR4, thus plasminogen activators cannot access the activation loop. Kringle domain is well
characterized by the role to bind lysine residues present in other peptide chains via a hydrogen bond. A Crystallographic study (64,67) demonstrated that in plasminogen, Lys50, Arg68 and Arg70 present on the N-terminal PAN domain bind to the lysin-binding sites of KR4 (Asp_{411} and Asp_{413}) and KR5 (Asp_{516} and Asp_{518}) to keep the closed conformation of plasminogen. The lysine residues present in fibrin can deprive the bindings of KRs from PAN, and lead to the relaxed conformation. Several functional studies shows KR5 has relatively high affinity to fibrin and is essential for fibrin to enhance the plasminogen activation mediated by plasminogen activators (68,69). The crystallographic study also shows KR5 have relatively a large mobility compared to the other kringles. Therefore it is predicted that fibrin-mediated conformational shift of plasminogen is initiated by the binding of KR5 with fibrin, which is followed by a large conformational change.

1.3 SMTPs

1.3.1 Origin and molecular characteristics

SMTPs (named after *Stachybotrys microspora* triprenyl phenols) are a family of small molecules produced by the fungus *S. microspora* (70) (Fig. 1.3). The SMTP molecule consists of a tricyclic $\gamma$-lactam moiety, an isoprene side chain and an $N$-linked side chain. Most of the congeners differ in the $N$-linked side chain moiety. The $N$-linked side chain can be derived from an amine present in the culture medium, and this finding enabled selective, efficient production of SMTP congeners through an amine-feeding cultivation of *S. microspora* (71). We recently identified a precursor of SMTPs (pre-SMTP) from the culture extract. Pre-SMTP nonenzymatically reacts with primary amines in both aqueous and organic solvents at a mild condition. This technique enabled the production of novel SMTP congeners which have not been able to obtain from the amine-feeding cultivation method (72). Our previous studies identified 44 SMTP congeners with different $N$-linked side chain moieties (71-80).
1.3.2 Plasminogen modulation

SMTPs enhance Glu-plasminogen binding to fibrin (73). These bindings are mediated by the lysine-binding sites, because a lysine analog inhibits the bindings. The two SMTPs activities, the elevation of Glu-plasminogen-fibrin binding and the promotion of Glu-plasminogen activation, are observed at the same range of SMTPs concentrations (81). Thus, a common mechanism may govern those actions. The fact that the activation of Glu-plasminogen is a conformationally regulated process is the key to understand this mechanism. The effect of SMTPs on the activation of Lys-plasminogen, which adopts an open conformation, is less prominent than its effect on Glu-plasminogen. Similarly, effects of SMTPs on Glu-plasminogen activation are reduced in the presence of the lysine analog 6-aminohexanoic acid or fibrinogen fragments, both of which relax Glu-plasminogen conformation. The molecular elution time of both Glu-plasminogen and Lys-plasminogen is slightly but significantly shortened in the presence of SMTPs. These results support the idea that the conformational status of plasminogen is responsible for SMTPs' effects to make the molecule susceptible to proteolytic activation and to binding to cells and fibrin. The reason why SMTPs are more effective on Glu-plasminogen rather than on Lys-plasminogen can be explained by the initial conformational status of plasminogen (64). With respect to the conformational modulation that leads to an elevated Glu-plasminogen activation, the SMTPs effect is similar to that of lysine analogs, whereas the effects of each compound on plasminogen binding are quite different. This implies that SMTPs acts as a plasminogen modulator that works through a mechanism distinct from the lysine-binding site (or AH site) occupancy. The action of SMTPs is shown schematically in Fig. 1.4. Plasmin formation in the presence of SMTP is transient in an incubation of Glu-plasminogen with u-PA. A decrease in plasmin activity follows a rapid increase in plasmin formation. This is because of autoproteolytic degradations of the catalytic domain of the plasmin molecule (70,82).
1.3.3 Therapeutic effects against acute ischemic stroke

SMTP-7 reduces cerebral infarct volume, edema, hemorrhagic transformation, and neuronal deficiency in AIS models of rodents and non-human primates (83-86). These therapeutic effects are accompanied by the promotion of plasmin formation and clot clearance (87), thus SMTP-7 is likely to have a plasminogen modulation activity in vivo. Surprisingly, SMTP-7 shows excellent therapeutic effects in severe conditions such as a thrombotic stroke model with delayed administrations by more than 3 hours or a severe embolic stroke model, where t-PA is not beneficial (84,88). The difference between SMTP-7 and t-PA is explained by the unique properties of SMTP-7 shown in reperfused brains: reducing expression of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6), adhesion molecules (I-CAM, V-CAM, and E-selectin), and tissue-degenerative protease (MMP-9), and reducing leukocyte adhesion to the damaged neurovascular endothelium (84,85). As described above, t-PA is conversely pro-inflammatory and tissue-degenerative, and worsens reperfusion injury. This notion raises a new question that how SMTP-7 produces such tissue-protective effects. One explanation previously made was that the phenolic hydroxyl group present in the tricyclic moiety of SMTP-7 serves as an anti-oxidant to reduce the oxidative stress causing reperfusion injury (89). The SMTPs actually act as radical scavengers in vitro (71), however, it is unclear whether the neuroprotective effect of SMTP-7 is caused only by this anti-oxidative activity. The beneficial properties of SMTP-7 prompted the development of this compound as an alternative stroke drug that can treat patients who do not benefit from the t-PA-based therapy. Further studies to prescribe the precise mechanism of the therapeutic effect of SMTP-7 are needed for its development.
Chapter 2 Identification of sEH as an Anti-inflammatory Target of SMTPs

This research was originally published in The Journal of Biological Chemistry: Matsumoto N, Suzuki E, Ishikawa M, Shirafuji T, and Hasumi K. Soluble epoxide hydrolase as an anti-inflammatory target of the thrombolytic stroke drug SMTP-7,

2.1 Introduction and aim

As mentioned above, t-PA influences the severity of post-stroke brain inflammation via reoxygenation and the activation of proteases participating in the fibrinolytic system. It was unclear whether the anti-inflammatory effect of SMTP-7 is independent of its plasminogen modulation activity. Here, we tried to elucidate the detailed anti-inflammatory mechanism of SMTP-7.

2.2 Results

2.2.1 Anti-inflammatory action independent of plasminogen modulation

Although SMTP-7 reduces inflammatory responses in thromboembolic stroke models, it was unclear whether or not this outcome is a consequence of the recanalization by thrombolytic enhancement. We thus employed inflammatory disease models (Guillain-Barré syndrome, ulcerative colitis, and Crohn’s disease models) that were irrelevant to thromboembolic complications to directly assess the anti-inflammatory action of SMTP-7. We compared the efficacy of SMTP-7 with that of its congener, SMTP-44D (Fig. 2.1A) (71), which is essentially inactive in the plasminogen modulation activity (Fig. 2.1B). In the Guillain-Barré syndrome model in rats, SMTP-7 (10 mg kg\(^{-1}\)) and SMTP-44D (10 mg kg\(^{-1}\)) both ameliorated neuritis symptoms as did the clinically used immunoglobulin formulation (400 mg kg\(^{-1}\)) (Fig. 2.1C). Both SMTP-7 (10 mg kg\(^{-1}\)) and SMTP-44D (10 mg kg\(^{-1}\)) alleviated the disease-associated body weight loss, stool inconsistency, and intestinal bleeding in the models of ulcerative colitis and Crohn’s disease in mice (Fig. 2.1D–G and H–K). These effects were comparable to or more prominent than the effects of the standard drug 5-ASA (100 mg kg\(^{-1}\)) and those of prednisolone (20 mg kg\(^{-1}\)). Thus, the anti-inflammatory action of SMTP is independent of the plasminogen modulation activity.
Figure 2.1
FIGURE 2.1. **Both SMTP-7 and SMTP-44D, a thrombolytically inactive congener, are effective in treating inflammatory disease in rodent models.**

A. The structures of SMTP-7 and SMTP-44D. B. Plasminogen modulation activity. The figure was reproduced based on published data (80). C. Efficacy in a rat Guillain-Barré syndrome model. EAN score: experimental autoimmune neuritis score. D-G. Efficacy in a mouse ulcerative colitis model. DAI score: disease activity index score. H-K. Efficacy in a mouse Crohn’s disease model. SMTP-7, 10 mg kg⁻¹; SMTP-44D, 10 mg kg⁻¹; 5-ASA, 100 mg kg⁻¹; prednisolone, 20 mg kg⁻¹; sulfonated immunoglobulin formulation, 400 mg kg⁻¹. *, P < 0.05; **, P < 0.01 by Dunnet’s test in comparison with control. #, P < 0.05 by Mann-Whitney’s U-test in comparison with control. Each value represents the mean ± SD obtained from 5 animals.

2.2.2 **Identification of sEH as a target**

To identify the molecule involved in the anti-inflammatory mechanism of SMTP, we designed an affinity matrix that contained an essential part of SMTP, tricyclic γ-lactam with a geranylmethyl side-chain (Fig. 2.2A). To prepare the SMTP congener with a primary amine at the N-linked side chain (SMTP-50), we first synthesized a precursor with the primary amine of SMTP-50 protected by a 9-fluorenylmethyloxycarbonyl group (SMTP-47) (Fig. 2.2A) by the precursor amine-fed culture method (78). After eliminating the protective group, the resulting SMTP-50 was coupled to cross-linked agarose beads.

Detergent-solubilized homogenates of the mouse liver were subjected to affinity chromatography on the SMTP-coupled beads. The resulting eluate gave several protein bands on SDS-polyacrylamide gel electrophoresis. We subjected four specifically eluted protein species to peptide-mass fingerprinting analysis (Fig. 2.2B), and every protein species was identified as a full-length form or a fragment of soluble epoxide hydrolase (sEH) (Fig. 2.2C and D, and Table S1). The purification of sEH to homogeneity was achieved when SMTP-affinity chromatography was performed using a cytosol fraction of detergent-free homogenates and an elution buffer containing AUDA, a competitive inhibitor for the epoxide hydrolase activity driven by the C-terminal domain of sEH (Cterm-EH) (Fig. 2.2E) (90).
FIGURE 2.2. Identification of sEH as an intracellular target of SMTPs. A, The synthesis of SMTP-50-coupled affinity matrix. We first synthesized SMTP-47 by the precursor amine-fed culture of S. microspora IFO 30018 using \(N\)-L-Fmoc-ornithine as a feeding amine. The Fmoc group was removed to afford SMTP-50, which was then coupled with \(N\)-hydroxysuccinimidy activated cross-linked agarose, affording SMTP-50-coupled affinity matrix. B, SDS-polyacrylamide gel electrophoresis of the eluates from the affinity chromatography of mouse liver homogenates. Arrowheads denote the protein bands specifically bound to the affinity matrix, and asterisks denote nonspecifically bound protein bands as judged by comparison with the results obtained with control matrix. C, Summary of the peptide mass fingerprinting analysis of protein bands 1–4. D, Results of the peptide mass fingerprinting analysis of protein band 1. The bars are for the found peptides cover the peptide sequences in Table 1. E, SDS-polyacrylamide gel electrophoresis of the preparation specifically eluted with AUDA by SMTP-affinity chromatography.
2.2.3 Inhibition of sEH

SMTP-7 inhibited both the Cterm-EH and the phosphatase activity driven by the N-terminal domain of sEH (Nterm-phos) (their respective IC₅₀ values were 23 ± 1 and 6 ± 1 µM) when we used the synthetic substrates PHOME (91) and AttoPhos (92), respectively (Fig. 2.3A and B). Similarly, SMTP-44D inhibited Cterm-EH and Nterm-phos (IC₅₀ 27 ± 2 and 24 ± 3 µM, respectively) (Fig. 2.3A and B). In addition, the structurally simplest congener SMTP-0 (which lacks the N-linked side chain) was inhibitory to both activities (IC₅₀ 6 ± 1 and 14 ± 1 µM, respectively) (Fig. 2.3A and B). Thus, the structural requirement for sEH inhibition is clearly distinguishable from that for plasminogen modulation activity, in which the N-linked side chain plays a crucial role (71,79).

We performed detailed kinetic analyses of sEH inhibition using SMTP-0 and the natural substrate 14,15-EET (93). The use of SMTP-0 was to avoid complexity of data analysis: SMTP-0 consists of a single core unit of SMTP (Fig. 2.3), whereas SMTP-7 has two core units that are asymmetrically configured (Fig. 2.1A), and each of these may differently interact with the enzyme. The kinetic results revealed a positive cooperativity for the hydrolysis of 14,15-EET (Fig. 2.4A) (Hill coefficient of 1.9 for the substrate binding; Fig. 2.4B), suggesting an allosteric interaction between the two monomers of sEH. The data were therefore analyzed based on a nonlinear mathematical model that allowed allosteryicity between the two equivalent catalytic sites of Cterm-EH (Fig. 2.4C). The pattern of the Cterm-EH inhibition by SMTP-0 fitted well to a competitive model (Fig. 2.4A and C). Moreover, Cterm-EH inhibition by SMTP-0 was competed for by AUDA (this class of inhibitor binds to the catalytic site in Cterm-EH (94)) (Fig. 2.4D and E). These results are consistent with the observation that sEH is specifically eluted with AUDA in SMTP-affinity chromatography. Regarding the Nterm-phos, the kinetic data fitted to a linear mathematical model (Fig. 2.5A–C), suggesting no cooperativity between the two Nterm-phos domains (Hill coefficient of 0.91; Fig. 2.5B). The inhibition of Nterm-phos by SMTP-0 was pseudo-noncompetitive with respect to the substrate AttoPhos (Fig. 2.5A and B). The pseudo-noncompetitive mechanism suggests that the Nterm-phos inhibition is mediated by the SMTP-0 binding to an allosteric site other than the substrate binding site in the Nterm-phos. Since there remained a possibility that the binding of SMTP-0 to the substrate binding site in the Cterm-EH might affect the activity of the Nterm-phos, we assessed the inhibitory activity of SMTP-0 toward the Nterm-phos in the presence of AUDA, which competed with SMTP-0 for binding to the catalytic site in the Cterm-EH (Fig. 2.4D). As a result, the presence of AUDA did not affect the SMTP-0 inhibition of the Nterm-phos (Fig. 2.5D and E). Thus,
SMTP-0 should bind to two distinct sites in sEH: one is the catalytic site in the Cterm-EH, and the other is an allosteric site that affects the Nterm-phos. The variability of the inhibition selectivity (IC₅₀ for the Cterm-EH vs. that for the Nterm-phos) supports the idea that SMTPs bind to two distinct sites in sEH (see Chapter 3 and Fig. 3.3).

FIGURE 2.3. Inhibition of sEH by SMTPs. Cterm-EH (A) and Nterm-phos (B) were determined in the presence of the indicated concentrations of SMTP-0, SMTP-7, and SMTP-44D using the following concentrations of enzyme and substrate: 4.7 nM sEH and 12.5 µM PHOME for Cterm-EH and 2.3 nM sEH and 5 µM AttoPhos for Nterm-phos. Each value represents the mean ± SD from triplicate determinations. The structure of SMTP-0 is shown.
Figure 2.4
FIGURE 2.4. Kinetic analysis of Cterm-EH inhibition by SMTP-0. A, Kinetic analysis of the Cterm-EH inhibition by SMTP-0 determined using 14,15-EET. Dashed lines represent the theoretical lines derived from the mathematical model in panel (C). B, Hill plots of the control data in panel (A). C, A diagram of the equilibrium in which allostery for the substrate binding between the two equivalent catalytic sites of the Cterm-EH is allowed (since sigmoidal velocity curves with a Hill coefficient of 1.9 that suggested an allosteric kinetic model for the substrate binding). As sEH consists of two identical monomers (E1 and E2), we hypothesized that the equilibrium substrate dissociation constants \( K_{S1} \), the primary substrate dissociation constant, and \( K_{S2} \), the secondary substrate dissociation constant) for each subunit were identical. (This means that E1 and E2 are indistinguishable from each other in this model.) Allostery for the inhibitor binding to each monomer is not considered in this model. In addition, no change in the dissociation constant for the substrate binding to the remaining vacant site is taken into account. S, substrate (14,15-EET); I, inhibitor (SMTP-0); P, product (14,15-DHET); \( K_I \), inhibitor dissociation constant. The equation is derived from the model. \( v \), reaction velocity; \( V_{\text{max}} \), maximum reaction velocity. To determine each constant, we tried various combinations of kinetic parameters to fit the velocity results to the equation. Among the parameters tested, the most probable values obtained were: \( V_{\text{max}} \) 11.5 µmol min\(^{-1}\) mg\(^{-1}\); \( K_{S1} \) 59 µM; \( K_{S2} \) 0.21 µM; \( K_I \) 21.2 µM; \( r^2 \) 0.99. D, Competition of SMTP-0 with AUDA in the Cterm-EH inhibition. The inhibition of Cterm-EH by SMTP-0 was determined with 4.7 nM sEH and 12.5 µM PHOME in the presence of the indicated concentrations of AUDA. Inset shows the secondary plots. Dashed lines represent the theoretical lines derived from the mathematical model in panel (E) fitted to the experimental data. E, A diagram of the equilibrium in which two inhibitors (SMTP-0, I\(_1\) and AUDA, I\(_2\)) competes with each other for binding to the catalytic site in an enzyme (Cterm-EH, E). S, substrate (PHOME); P, product; \( K_{I1} \), inhibitor dissociation constant for I\(_1\); \( K_{I2} \), inhibitor dissociation constant for I\(_2\). The equation is derived from the model. \( v \), reaction velocity; \( V_{\text{max}} \), maximum reaction velocity. To determine each constant, we fitted the velocity results obtained in the presence of 5 µM of S (PHOME) with various concentrations of I\(_1\) and I\(_2\) to the equation using the value of \( V_{\text{max}} \) and \( K_S \) obtained in the absence of the inhibitor \( (V_{\text{max}} \ 1.8 \times 10^4 \ \Delta \text{fluorescence min}^{-1}, \ K_S \ 59 \ \mu\text{M}) \). The values obtained were: \( K_{I1} \) 6.8 µM; \( K_{I2} \) 6.3 nM; \( r^2 \) 0.97. Each value represents the mean ± SD from triplicate determinations.
FIGURE 2.5. Kinetic analysis of Nterm-phos inhibition by SMTP-0. A, Kinetic analysis of the Nterm-phos inhibition by SMTP-0 determined using AttoPhos. Dashed lines represent the theoretical lines derived from the mathematical model in panel (C). B, Hill plots of the control data in panel (A). C, A diagram of the equilibrium in which no allostericity for the substrate binding between the two equivalent catalytic sites of the Nterm-phos is accounted (this was because hyperbolic velocity curves with a Hill coefficient of 0.91 was obtained). Also, allostericity for the inhibitor binding to each monomer is not considered. E, Ntem-phos; S, substrate (AttoPhos); I, inhibitor (SMTP-0); P, product; $K_S$, substrate dissociation constant; $K_{I-S}$, dissociation constant between EI complex and S; $K_I$, inhibitor dissociation constant. The equation is derived from the model. $v$, reaction velocity; $V_{max}$, maximum reaction velocity. To determine each constant, the velocity results obtained with various concentrations of I and S were fitted to the equation. The values obtained were: $V_{max}$ 230 Δfluorescence min$^{-1}$; $K_S$ 2.1 μM; $K_{I-S}$ 2.7 μM; $K_I$ 12 μM; $r^2$ 0.98. D, Absence of competition of SMTP-0 with AUDA in the Nterm-phos inhibition. The inhibition of Nterm-phos by SMTP-0 was determined with 2.3 nM sEH and 5 μM AttoPhos in the presence of the indicated concentrations of AUDA. The green dashed line represents the theoretical line that considers the competition of SMTP-0 with AUDA derived from a model in which the inhibition of Nterm-phos by SMTP-0 is competitively affected by AUDA. Experimental data demonstrated that AUDA did not affect the activity of the Nterm-phos. Each value represents the mean ± SD from triplicate determinations.
2.2.4 Inhibition of EET metabolism

HepG2 cells had an ability to hydrolyze 14,15-EET to 14,15-dihydroxyeicosatrienoic acid (DHET), an inactive diol. SMTP-7 inhibited the formation of 14,15-DHET from 14,15-EET added to the culture medium (IC\textsubscript{50} 6.5 µM) (Fig. 2.6A). Along with the inhibition of the 14,15-EET hydrolysis, the level of 14,15-EET was elevated in the presence of SMTP-7 (Fig. 2.6A). SMTP-0 and SMTP-44D were also active in inhibiting 14,15-DHET formation from 14,15-EET in HepG2 cells (IC\textsubscript{50} 1.2 and 9.2 µM, respectively) (Fig. 2.6B).

FIGURE 2.6. Inhibition of 14,15-EET hydrolysis in HepG2 cells in culture. A and B, Inhibition of 14,15-EET hydrolysis in HepG2 cells by SMTP-7 (A), SMTP-0 and SMTP-44D (B). Each value represents the mean ± SD from triplicate determinations.

2.2.5 Effects on the level of arachidonate-derived lipid mediators in plasma from Guillain-Barré syndrome model rats

To confirm the impact of sEH inhibition by SMTP-7 and its selectivity on, we measured the levels of metabolites in the cyclooxygenase, lipoxygenase, and cytochrome P450 pathways using plasma obtained from Guillain-Barré syndrome model rats. A global analysis revealed that SMTP-7 did not significantly affect the levels of 47 out of 48 metabolites examined (see Experimental Procedures for metabolites analyzed), resulting in a small change in the distribution of the three classes of arachidonate metabolites (Fig. 2.7A). The only one metabolite that was significantly affected by the treatment with SMTP-7 was 11,12-DHET. The level (% distribution among the 48 metabolites) of 11,12-DHET in SMTP-7-treated rats was significantly lower than that in saline-treated rats (13.5 ± 0.17% compared to 20.2 ± 0.04% in saline group, P < 0.05; Fig. 2.7B). The levels of 5,6-, 8,9-, and 14,15-DHETs, however, were not significantly changed by the treatment with SMTP-7 (Fig. 2.7B). The levels of all regioisomers of EETs were too low to be detected by the method employed. The
result that SMTP-7 selectively decreased 11,12-DHET can partly be explained by inhibition of sEH. The interpretation of this result is described in Discussion.

FIGURE 2.7. Global analysis of arachidonate-derived lipid mediators in plasma derived from Guillain-Barré syndrome model rats. A, Distribution of the arachidonate-derived lipid mediators as categorized by the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) pathways. The total of the levels of 48 metabolites tested (see Experimental Procedures) in each group (saline-treated or SMTP-7 treated) was taken as 100%. B, Effects of SMTP-7 treatment on the levels of 5,6-, 8,9-, 11,12, and 14,15-DHETs. *, $P < 0.05$ by unpaired Student’s $t$-test. Each value represents the mean ± SD obtained from 4 determinations.
Chapter 3 Structure-Activity Relationships of SMTPs with Respect to Plasminogen Modulation and sEH Inhibition

3.1 Introduction and aims

SMTP-7 shows excellent therapeutic effects against AIS via its dual action: enhancing clot-clearance and reducing pro-inflammatory responses (51,84-87,95). These effects were demonstrated to be caused by two distinct mechanisms: plasminogen modulation (64,70,73,81) and the competitive inhibition of C-terminal epoxide hydrolase activity of sEH (Cterm-EH) (see section 2.1 and the discussion). SMTP-7 has been identified as a most potent plasminogen modulator among more than 44 SMTP congeners (71-80), but it is unclear whether SMTP-7 is also a potent Cterm-EH inhibitor. If we can find the congeners possessing potent activities for both plasminogen modulation and Cterm-EH inhibition, they may show excellent therapeutic effect to AIS at a lower dose than that of SMTP-7, contributing to the prevention of the toxic effects of the agents. Therefore, first in this section, we assayed SMTP congeners, which have various N-linked side chains, for Cterm-EH inhibition, and combined these results with their plasminogen modulation activities (51,84-87,95) to find the congeners promising for excellent therapeutic potentials.

In addition to plasminogen modulation and Cterm-EH inhibition, SMTPs also non-competitively inhibits N-terminal phosphatase activity of sEH (Nterm-phos) (see section 2.1). This Nterm-phos inhibition is mediated by another, unidentified binding site of sEH rather than that for Cterm-EH inhibition. There is no clear consensus as to the physiological role of Nterm-phos, while several studies using genetic manipulations demonstrated its role in cholesterol homeostasis (96) and vascular functions (97). To date, lipid sulfates and sulfonates (98), a sesquiterpene derivative N-acetyl-S-farnesyl-L-Cysteine (97), and a cysteine alkylating agent ebselen (92) are identified as Nterm-phos inhibitors, however, their intracellular activities and specificities are doubtful. N-acetyl-S-farnesyl-L-Cysteine and ebselen, at least, have other target proteins at the same ranges of concentrations (99,100). This lack of the specific inhibitor is a major limitation of studies on Nterm-phos. Therefore, second in this section, we assayed SMTP congeners for Nterm-phos inhibition to elucidate structure-activity relationships, to contribute to future investigations for the physiological role of Nterm-phos.

3.2 Results

3.2.1 Plasminogen modulation

We synthesized new SMTP congeners to examine various N-linked side chains for their activity against plasminogen, Cterm-EH, and Nterm-phos. The plasminogen modulating activities were assessed for the 8 SMTP congeners (6 of which were newly synthesized here
[SMTP-52, SMTP-57, SMTP-58, SMTP-60, SMTP-61, and SMTP-62], and the other 2 were previously identified but not characterized [SMTP-54, SMTP-55] (72)) as the activity enhancing plasminogen activation catalyzed by u-PA. As a result, plasminogen modulating activity was significantly detected only in SMTP-61 (Fig. 3.1). The concentration giving 10-fold enhancement (EC_{10}) and maximum enhancement (E_{max}) of SMTP-61 was 110 μM and 16-fold, respectively. The plasminogen modulation index (the ratio E_{max}/EC_{10}) of 0.15-fold μM^{-1} of SMTP-61 is ~1/11 of that of SMTP-7 (1.57-fold μM^{-1}) (76), one of the most potent SMTP congeners. These results are consistent with the notion established in the previous studies that potent plasminogen modulating activity requires both an aryl moiety and an acidic group (71,76).

![Plasminogen modulation activity of newly produced SMTPs](image)

FIGURE 3.1 Plasminogen modulation activity of newly produced SMTPs. Plasminogen activation mediated by u-PA were assessed in the presence of indicated concentration of each SMTP congener. The concentrations giving 10-fold enhancement (EC_{10}) and extents of maximum enhancement (E_{max}) were determined for each SMTP congener. E_{max} and the reciprocal of EC_{10} are independent indexes that represent the potency of the compound tested. The ratio E_{max}/EC_{10} is introduced to represent comprehensive potency. NA, not available (due to that enhancement did not reach 10-fold at concentrations tested).
3.2.2 Structure-activity relationships with respect to plasminogen modulation and Cterm-EH inhibition

Although 7 of 8 currently evaluated SMTP congeners were inactive in plasminogen modulation, some of them were effective in inhibiting Cterm-EH of sEH (Fig. 3.2A, left panel). SMTP-61 and -62 were inhibitory to Cterm-EH (IC_{50} 36 and 48 μM, respectively), whereas SMTP-58 and -60, the analogs that lacked the side-chain carboxyl or hydroxyl group, were essentially inactive (IC_{50} > 100 μM). Similarly, SMTP-34, -38, and -57, which do not have a negatively ionizable side-chain, were much less active (Fig. 3.2A, left panel). The only exception was SMTP-0, which lacks the N-linked side-chain. SMTP-0 (IC_{50} for Cterm-EH = 6 μM) was one of the potent congeners tested. Other congeners with a negatively ionizable group at the N-linked side-chain were generally inhibitory to Cterm-EH (SMTP-4, -5, -6, -13, -14, -18, 19, -23, -24, -25, -42, -43, -44, and -44D). Of these, congeners with relatively high plasminogen modulation activity with E_{max}/E_{10} > 0.4-fold μM^{-1} (SMTP-14, -19, -21, -23, -25, -43, and -43D), only SMTP-21 was weak in Cterm-EH inhibition (IC_{50} > 60 μM) (Fig. 3.2A, left panel).

These results clearly demonstrate that plasminogen modulation and Cterm-EH inhibition require distinct structural feature of the N-linked side-chain. Although an N-linked side-chain with an aromatic and a negatively ionizable groups is essential for plasminogen modulation, only the core unit of SMTP (a tricyclic γ-lactam with a geranylmethyl group) is sufficient for the inhibition of Cterm-EH. However, certain structural feature of the N-linked side-chain, such as the presence or absence and of carboxyl or hydroxyl group as well as its position, can modulate the potency of Cterm-EH inhibition without affecting plasminogen modulation as typically observed with SMTP-58 -60, and -62 (Fig. 3.2A, left panel).

3.2.3 Structure-activity relationships with respect to plasminogen modulation and Nterm-phos inhibition

Many of the congeners with little or no plasminogen modulation activity (E_{max}/E_{10} < 0.1-fold μM^{-1}) were inhibitory to Nterm-phos (IC_{50} < 50 μM) (SMTP-4, -5, -11, -12, -18, -44, -57, -58, and -60) as did all the congeners with relatively potent plasminogen modulation activity (E_{max}/E_{10} > 0.4-fold μM^{-1}) (SMTP-14, -19, -21, -23, -25, -43, and -43D) (Fig. 3.2A, right panel). The congeners with little or no inhibitory activity toward Nterm-phos (IC_{50} > 50 μM) were SMTP-34, -38, -52, and -62. All of these were inactive in plasminogen modulation. In summary, the structural requirement for plasminogen modulation and Nterm-phos inhibition is apparently different, and there are several congeners potently inhibit Nterm-phos without plasminogen modulation activity (SMTP-4 and -5). In general, congeners with high
plasminogen modulation activities ($E_{\text{max}}/EC_{10} > 0.9\text{-fold } \mu M^{-1}$) (SMTP-19, -25, and -43) are effective inhibitors of Nterm-phos, while the potencies of SMTP-14 and -21 were low (Fig. 3.2A, right panel).

3.2.4 Structure-activity relationships with respect to Cterm-EH and Nterm-phos inhibition in combination of plasminogen modulation

The above-mentioned results were summarized in Fig. 3.2B for comprehensive analysis. SMTP-7, -19, and -25 are active among the three activities, plasminogen modulation and inhibitions of Cterm-EH and Nterm-phos. SMTP-0, and -26 are inhibitory to both Cterm-EH and Nterm-phos of sEH but inactive in plasminogen modulation. SMTP-5D and -43D have significant activities in plasminogen modulation and Nterm-phos inhibition but very weak in Cterm-EH inhibition. SMTP-54 and -55 are inhibitory to Cterm-EH but very weak in Nterm-phos inhibition and plasminogen modulation. SMTP-58 and -60 are inactive in plasminogen modulation and Cterm-EH inhibition but inhibitory to Nterm-phos. SMTP-16, -33 and -38 are totally inactive in the three parameters.
FIGURE 3.2 Structure-activity relationships of SMTP congeners with respect to plasminogen modulation and inhibitions of Cterm-EH and Nterm-phos of sEH. A, Structure-activity relationships with respect to plasminogen modulation and Cterm-EH (left panel) and to plasminogen modulation and Nterm-phos (right panel). The number in circle represents the SMTP number. The colors of circle represent congeners as follow: pale olive, new ones; dark blue, previously characterized ones; dark red, newly characterized ones. Characteristic congeners are encircled in a dashed line. B, Structure-activity relationships with respect to sEH inhibition in combination of plasminogen modulation. Cterm-EH inhibition is plotted against Nterm-phos inhibition with numbered symbols as in panel a. The colors indicate the potency of plasminogen modulation activity as shown in the right bar. Characteristic congeners are encircled in a dashed line. C, Structures of the N-linked side-chain of the congeners tested are shown. Red “R” represents the core structure shown in Fig. 3.1.
Chapter 4 Discussion and Conclusion

Currently, only t-PA is recommended for the treatment of AIS, however, is used only in 1-9% of all AIS patients, due to the narrow therapeutic-time window and the promotion of hemorrhagic transformation (44,45). SMTPs is a series of fungal metabolites (70). SMTP-7, a congener of SMTPs, promotes clot clearance and thus shows a therapeutic action against animal models of AIS (83-87). The mechanism of action is explained by the plasminogen modulation activity of SMTP-7, which enhances the activation of plasminogen, the fibrinolytic plasma protease, by plasminogen activators (73). Unexpectedly, SMTP-7 also reduced hemorrhagic transformation and had a wide therapeutic-time window (51,83,101). These favorable actions were associated with anti-inflammatory effects of SMTP-7 (51,84,85,89). This finding has motivated us to develop SMTP-7 as a novel therapeutic agent for AIS, which can be used in a broad spectrum of AIS patients. Here we study on the anti-inflammatory mechanism of SMTP-7.

First we identified sEH as an anti-inflammatory target of SMTP-7. As the fibrinolytic system can modulate tissue inflammation in several ways (see introduction), it had been unclear whether the anti-inflammatory effects of SMTP-7 seen in AIS models are independent of its plasminogen modulation activity. Thus we performed the animal experiments using SMTP-7 and SMTP-44D (the SMTP congener which does not have a plasminogen modulation activity (80)) and the inflammatory disease models (Guillain-Barré syndrome, ulcerative colitis, and Crohn's disease) which are apparently irrelevant to haemostatic/fibrinolytic systems. These experiments showed therapeutic effects in both SMTP-7 and SMTP-44D, thus the existence of another anti-inflammatory target was demonstrated. These results also demonstrated that the chemical structure shared within SMTP-7 and SMTP-44D (the core structure and the carboxylic group of the \( \text{N} \)-linked side chain) is the crucial part for the anti-inflammatory effects. Thus we performed affinity-based target purification using the matrix which the core moiety of SMTPs including the carboxylic group of the \( \text{N} \)-linked side chain is immobilized on, and found sEH as the major protein binding on this SMTP-matrix. This matrix also enabled us to obtain highly purified sEH preparation, which was used in the subsequent biochemical experiments. sEH is a bifunctional enzyme possessing Cterm-EH activity and Nterm-phos activity (102,103), and present in many kinds of tissues and organs (104), including a brain (105). Cterm-EH hydrolyzes endogenously produced anti-inflammatory epoxy fatty acids, such as EETs (93), to inactive dihydroxy derivatives. We found that SMTP-0, SMTP-7, and SMTP-44D inhibited both Cterm-EH and Nterm-phos \textit{in vitro}. Biochemical analysis were performed with SMTP-0,
because SMTP-0 is the core structure itself which all SMTP congeners share (78), whereas SMTP-7 has an asymmetric structure composed of two core structures (76), and may bind to sEH in two different poses, which would be complex too much for a mathematical analysis. The inhibitory mechanisms of SMTP-0 were estimated as competitive for Cterm-EH and noncompetitive for Nterm-phos. We observed a positive cooperativity in Cterm-EH, whereas no cooperativity (93) or a negative cooperativity in Cterm-EH (105) has been reported in previous studies. These variable findings may be due to the use of enzyme from different sources (native or recombinant) and/or buffer compositions. As expected from the crystal structure, sEH has higher order intradomain and interdomain interactions (106), and environmental conditions would affect the conformational status of sEH to exhibit cooperativity. Nevertheless, kinetic parameters obtained with these three investigations are relatively close [V\text{MAX} 11.5 \mu\text{mol min}^{-1} \text{mg}^{-1} and apparent K_M [(K_{S1} \times K_{S2})^{1/2}] 3.52 \mu\text{M} in this study (Fig. 2.4); V\text{MAX} 9.0 \mu\text{mol min}^{-1} \text{mg}^{-1} and K_M 4 \mu\text{M for 14(R),15(R)-EET, and V\text{MAX} 1.36 \mu\text{mol min}^{-1} \text{mg}^{-1} and K_M 5 \mu\text{M for 14(S),15(S)-EET by Zeldin et al. (105); V\text{MAX} 20 \mu\text{mol min}^{-1} \text{mg}^{-1} and apparent K_M [(K_{S1} \times K_{S2})^{1/2}] 6.3 \mu\text{M by Marowsky et al. (105)]. Thus we concluded our observation would have correctly reflected the nature of Cterm-EH. SMTP-7 reduced the hydrolysis of 14,15-EET to 14,15-DHET in the culture of HepG2 cells. In Guillain-Barré syndrome rats, SMTP-7 reduced plasma level of 11,12-DHET, whereas the levels of other 47 signal-mediating arachidonate derivatives (including 5,6-, 8,9-, and 14,15-regioisomers of DHET) were unaffected. The lack of effect of SMTP-7 on the levels of 5,6-, and 8,9-DHET can be explained by the low catalytic activity of sEH toward these regioisomers (93,107), letting sEH to contribute lesser to these levels. We speculate the lack of effect on the 14,15-DHET level is due to complex mechanisms of the formation/catabolism (degradation and excretion) of EETs and DHETs under physiological conditions. According to previous literatures (93,107,108) the specific activity of human sEH for the hydrolysis of 14,15-EET is approximately 2 times higher than that of 11,12-EET. In our animal model, the circulating level of 14,15-DHET was only 1.1 times higher than that of 11,12-DHET in control animals. Thus, it is likely that the rates of degradation and/or excretion of 14,15-DHET in this model is higher than those of 11,12-DHET. This may make it difficult to reflect the impact of sEH inhibition by SMTP-7. The selective change in the 11,12-DHET level by SMTP-7 suggested a specificity of SMTP-7 in the arachidonate metabolisms. Along with the in vitro data, this in vivo result supported the idea that sEH is an anti-inflammatory target of SMTP-7. The deficiency of sEH and the inhibition of the Cterm-EH have been reported to be protective against disease progression in animal models of inflammatory bowel
diseases (109) and AIS. Putting all these evidences together, the anti-inflammatory effects of SMTP-7 can be concluded to be due to its Cterm-EH inhibition and the following promotion of the internal level of anti-inflammatory epoxy-fatty acids.

The finding that SMTP-0, SMTP-7, and SMTP-44D inhibit both Cterm-EH and Nterm-phos encouraged us to investigate these structure-activity relationships and to search for congeners possessing excellent activity, using other SMTP congeners identified in the previous studies (71-80). As plasminogen modulation and Cterm-EH inhibition are demonstrated to cooperatively treat AIS, congeners possessing potent activities in both the plasminogen modulation and Cterm-EH inhibition are promising candidates for the novel therapeutic agents for AIS which are effective at a lower dose. The two-dimensional plot of the plasminogen modulation versus Cterm-EH inhibition suggested that $N$-linked side chains are normally unnecessary for Cterm-EH inhibition because SMTP-0, the simplest congener possessing only a hydrogen atom on its $N$-linked side chain (78), was the second most potent Cterm-EH inhibitor, whereas $N$-linked side chains are essential for the plasminogen modulation activity. SMTP-7, SMTP-19, SMTP-25, and SMTP-28 were potent in both activities among 39 congeners tested. Compared to SMTP-7, $IC_{50}$ for Cterm-EH was 5-folds smaller in SMTP-28, while the plasminogen modulation index was reduced 3-folds. An $N$-phenylcarboxylic acid, the structure shared among SMTP-19, SMTP-25, and SMTP-28 may be favorable for both plasminogen modulation and Cterm-EH inhibition, or taking the carboxylic group of SMTP-7 into account, the presence of a negatively charging moiety around the joint of a $N$-linked side chain may be favorable.

Currently no consensus is achieved on the physiological role of Nterm-phos among researchers, while three studies using genetic manipulations suggest its implication for the cholesterol homeostasis (110) and vascular functions (96,97). The lack of a specific inhibitor makes it difficult to perform biological studies on Nterm-phos. We found here that SMTP-0 noncompetitively inhibits Nterm-phos. This is the first identification of the noncompetitive inhibitor for Nterm-phos, while an allosteric competitive inhibitor and an irreversible competitive inhibitor were previously reported by Tran et. al. (98) and Morisseau et al. (92), respectively. Our findings may be useful to develop novel Nterm-phos inhibitors because the noncompetitive inhibition implies the existence of an allosteric site modulating Nterm-phos activity distinct from the substrate-binding site, which has a different structural requirement for inhibitors from that for competitive inhibitors. Then we examined the inhibitory activity of 39 SMTP congeners toward Nterm-phos to elucidate structure-activity relationships. Interestingly, SMTP-7 was the most potent Nterm-phos inhibitor among the congeners tested.
Nterm-phos inhibition might contribute to the bioactivity of SMTP-7. Congeners possessing an \( N \)-phenylcarboxylic acid as their \( N \)-linked side chains showed potent Nterm-phos inhibition.
Experimental procedures

Animal experiments

All of the animal protocols were approved by the institutional animal experiment committees at the Tokyo Noko University and Nihon Pharmaceutical. Male C57BL/6J mice (7 weeks old; Japan SLC, Hamamatsu) and male Lewis rats (6 weeks old; Charles River Laboratories Japan, Yokohama) were used after one week of preliminary rearing for the inflammatory disease models. Retired male ICR mice (Japan SLC, Hamamatsu) were used to obtain the livers for the sEH purification.

SMTP congeners

The SMTP congeners used in this study were produced by *S. microspora* as described previously (71,72,76-79). For *in vivo* studies, SMTP-7 and SMTP-44D were converted to respective sodium salts. SMTP-52, SMTP-57, SMTP-58, SMTP-60, SMTP-61, and SMTP-62 were produced by a single step reaction of pre-SMTP with primary amines (72). Briefly, 10 mg of pre-SMTP was dissolved in 1.25 ml of methanol, and was added to 4 ml of water/methanol/acetic acid (75:12.5:12.5) containing excess amounts of amines, and then the reactions were performed at 50°C for 2 hours. The amines used here are following: asparagine for SMTP-52, 2-aminopropane for SMTP-57, cyclohexylamine for SMTP-58, cyclohexylmethylamine for SMTP-60, 1-aminocyclohexanecarboxylic acid for SMTP-61, and *trans*-4-aminocyclohexanol for SMTP-62. Produced SMTPs were purified with reversed-phase HPLC. Physicochemical analysis for these SMTPs were performed as follows: mass, on JMS-T100LP (JEOL, Tokyo, Japan); UV, on U-2910 analyzer (HITACHI, Tokyo, Japan) with methanol as a solvent; optical rotation, on DIP-360 (JASCO, Tokyo, Japan) with methanol as a solvent; NMR, on JNM-alpha600 (JEOL, Tokyo, Japan) with acetone-\textit{d}_6 and methanol-\textit{d}_4 as solvents. Their physicochemical data and summery were shown in supplementary table 3 and supplementary figure 1-5. Chemical structures of produced SMTPs were proposed as shown in Fig. 3.1.

Guillain-Barré Syndrome Model

On day 0, male Lewis rats (7 weeks old) were given 0.1 ml of a 1:1 mixture of the synthetic P2 peptide (H-TESPFKNTESFKLGPQTEETTADNR-OH corresponding to T53-R78 of bovine P2 protein; 2.5 mg ml\textsuperscript{-1} in saline) (111) and Freund’s incomplete adjuvant containing *Mycobacterium tuberculosis* H37 Ra (DIFCO) by injection into foot pads of both of the hind limbs. Sodium salts of SMTP-7 and SMTP-44D dissolved in 5% (wt vol\textsuperscript{-1}) mannitol was intraperitoneally injected at a dose of 10 mg kg\textsuperscript{-1} through day 7 to 16. As a
Standard, sulfonated immunoglobulin formulation (Kaketsuken, Kumamoto, Japan) was given intravenously at a dose of 400 mg kg\(^{-1}\) on day 0, 7, 14, 15, and 16. Control animals received no drug treatment. Normal animals received neither P2 peptide nor any drug. The experimental autoimmune neuritis score (score 0, normal; 0.5, reduced tone of the tail; 1, limp tail; 2, moderate paraparesis; 3, severe paraparesis; 4, tetraparesis or death) was determined on day 7, 9, 11, 13, 15, 17, 20 and 24. There were 5 animals in each group.

**Ulcerative Colitis Model**

Male C57BL/6J mice (8 weeks old) were given dextran sulfate sodium salt (36–50 kDa) (112) contained in drinking water (2%, wt vol\(^{-1}\)) daily through day 0 to 7. Sodium salt of SMTP-7 or SMTP-44D was intraperitoneally injected at a dose of 10 mg kg\(^{-1}\). As standards, 5-aminosalicylic acid (5-ASA) (113) (10 mg ml\(^{-1}\) in 0.5% carboxymethylcellulose) or sodium prednisolone succinate (2 mg ml\(^{-1}\) in phosphate-buffered saline) were given orally at a dose of 100 mg kg\(^{-1}\) and 20 mg kg\(^{-1}\), respectively. Drugs were given daily through day 0 to 7. Control animals received no drug treatment. Normal animals received neither dextran sulfate nor any drug. The disease activity index (DAI) score (114,115), determined based on the change in body weight (score 0, <1%; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4: >20%), stool inconsistency (score 0, normal; 2, loose; 4, diarrhea), and stool blood (score 0, negative; 2, occult blood; 4, gross bleeding), was measured on days 2, 4, 5, 6, 7 and 8. There were 5 animal in each group.

**Crohn’s Disease Model**

Male C57BL/6J mice (8 weeks old) were intrarectally injected with 2,4,6-trinitrobenzene sulfonic acid (TNBS) (116) solution (20 mg ml\(^{-1}\) in 50% ethanol) at a dose of 100 mg kg\(^{-1}\). SMTP-7, SMTP-44D, 5-ASA, or prednisolone was administered as described for the dextran sulfate model. These treatments were made 30 min before the TNBS injection as well as 24, 48, and 72 h after the TNBS injection. Control animals received no drug treatment. Normal animals received neither TNBS nor any drug. The DAI score was measured on days 1, 2, 3, and 4 following the TNBS injection. There were 5 animal in each group.

**SMTP-47**

SMTP-47 was synthesized by the microbial amine feeding method (78). For the preparation of feeding amine, \(N_\alpha\)-Boc-\(N_\delta\)-Fmoc-L-ornithine (60 mg ml\(^{-1}\) in tetrahydrofuran) was treated with equal volume of trifluoroacetic acid, affording \(N_\delta\)-L-Fmoc-ornithine. The culture of *S. microspora* IFO 30018 (100 ml) was fed with 100 mg of \(N_\delta\)-L-Fmoc-ornithine, and the resulting SMTP-47 (89 mg) was purified by reverse-phase HPLC developed with MeOH/0.1% formic acid (85:15). \(^1\)H NMR (acetone-\(d_6\), 600 MHz): \(\delta\) 7.83 (2H, d, \(J = 7.3\) Hz),
7.67 (2H, d, J = 7.3 Hz), 7.38 (2H, m), 7.29 (2H, t, J = 7.3 Hz), 6.80 (1H, d, J = 2.2 Hz), 5.16 (1H, t, J = 6.6 Hz), 5.06 (1H, m), 4.97 (1H, dd, J = 4.4, 11.0 Hz), 4.44 (1H, d, J = 16.1 Hz), 4.31 (3H, m), 4.20 (1H, d, J = 6.6 Hz), 3.95 (1H, dd, J = 5.9, 7.3 Hz), 3.23 (2H, m), 3.02 (1H, dd, J = 5.9, 17.6 Hz), 2.68 (1H, dd, J = 7.3, 17.6 Hz), 2.20 (2H, m), 2.06 (4H, m), 1.95 (2H, m), 1.71 (2H, m), 1.62 (3H, s), 1.57 (3H, s), 1.56 (2H, m), 1.53 (3H, s), 1.28 (3H, s). 13C NMR (acetone-d$_6$, 150 MHz): $\delta$ 172.95, 169.71, 157.18, 157.13, 149.88, 145.18, 142.09, 135.70, 132.56, 131.65, 128.43, 127.87, 126.08, 125.19, 125.12, 121.77, 120.73, 112.76, 100.86, 79.93, 67.61, 66.68, 54.30, 48.15, 44.97, 40.90, 40.40, 38.42, 27.67 (2 signals overlapped), 27.64, 27.38, 25.80, 22.23, 18.79, 17.70, 15.99. MALDI-TOF MS (m/z): [M + Na]$^+$ calcd. for C$_{43}$H$_{50}$N$_2$NaO$_8$, 745.3465; found, 745.3575. UV (MeOH): $\lambda_{\text{max}}$ nm ($\varepsilon$) 208 (86,390), 263 (29,330), 289 (7,370), 300 (9,100). IR (neat): $\nu_{\text{max}}$ cm$^{-1}$ 3329, 3064, 2968, 2926, 1701, 1670, 1620, 1533, 1462, 1356, 1252, 1157, 1076, 847, 744, 542.

SMTP-50

SMTP-47 (90 mg) was treated with piperidine/N,N-dimethylformamide (6:1) for 1 h to afford SMTP-50, which was purified by reverse-phase HPLC developed with a linear gradient of MeOH in 0.1% formic acid (60–100%) for 20 min, yielding 30.3 mg of the purified material. $^1$H NMR (CD$_3$OD, 600 MHz): $\delta$ 6.74 (1H, s), 5.12 (1H, t, J = 6.9 Hz), 5.06 (1H, t, J = 6.9 Hz), 4.75 (1H, dd, J = 4.8, 10.2 Hz), 4.61 (1H, d, J = 16.8 Hz), 4.26 (1H, d, J = 17.4 Hz), 3.88 (1H, t, J = 6.0 Hz), 2.97 (3H, m), 2.64 (1H, dd, J = 7.2, 17.4 Hz), 2.16 (3H, m), 2.05 (2H, m), 1.96 (2H, m), 1.90 (1H, m), 1.68 (1H, m), 1.64 (3H, s), 1.61 (3H, m), 1.59 (3H, s), 1.57 (3H, s), 1.26 (3H, s). 13C NMR (CD$_3$OD, 150 MHz): $\delta$ 177.17, 171.83, 157.82, 150.01, 136.39, 132.66, 132.13, 125.39, 125.36, 122.24, 113.21, 100.91, 80.09, 68.22, 57.09, 45.92, 40.81, 40.01, 38.72, 28.61, 27.73 (2 signals overlapped), 25.89, 25.81, 22.53, 18.95, 17.76, 16.04. ESI-TOF-MS (m/z): [M + H]$^+$ calcd. for C$_{28}$H$_{41}$N$_2$O$_6$, 501.2965; found, 501.3003. UV (MeOH): $\lambda_{\text{max}}$ nm ($\varepsilon$): 216 (41,700), 260 (9,600), 300 (2,800). IR (neat): $\nu_{\text{max}}$ cm$^{-1}$ 3386, 3208, 2967, 2926, 1657, 1611, 1466, 1381, 1245, 1213, 1163, 1080, 1047. $[\alpha]_{D}^{25} = -12.1^\circ$ (c. 1.0, MeOH).

SMTP-50-Coupled Affinity Matrix

One column volume (1 ml for target identification or 5 ml for target purification) of 1.2 mM SMTP-50, dissolved in 10 mM sodium phosphate, pH 8.3, was applied to a HiTrap NHS-activated HP column (GE Healthcare, Buckinghamshire, UK) at room temperature for 30 min. The column was then treated with monoethanolamine to block residual N-hydroxysuccinimidyl group of the matrix.
Identification of SMTP-Binding Protein

The livers from male ICR mice were perfused with ice-cold saline and homogenized in 4 volumes of 25 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.2% (wt vol⁻¹) sodium deoxycholate (buffer A). A supernatant fraction was obtained after centrifugation at 1,000 g for 15 min, followed by 20,000 g for 15 min at 4°C. After filtration, 15 ml of the supernatant was applied to a 1-ml SMTP-affinity column pre-equilibrated with buffer A at 20°C. The column was washed with 10 ml of buffer A and developed with 10 ml of buffer A containing additional NaCl (500 mM, finally). Aliquots of the eluate were resolved by reduced SDS-polyacrylamide gel electrophoresis. Coomassie Brilliant Blue R250-stained protein bands were excised from the gel and digested with trypsin. The resulting peptides were subjected to chemically assisted fragmentation post-source decay analysis by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry on a Ettan MALDI-TOF Pro (Amersham Biosciences, Piscataway, NJ, USA) or LC-MALDI-TOF/TOF analysis on a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Detected masses were subjected to amino acid sequence analysis and to comparison with theoretical peptide masses on MASCOT search engine (Matrixscience, Boston, MA) to identify the protein species.

Purification of sEH by SMTP-Affinity Chromatography

All of the following operations were carried out at 0–4°C. The livers from male ICR mice were homogenized in 11.5 volumes of 76 mM sodium phosphate, pH 7.4. A supernatant fraction was obtained after centrifugation at 1,000 g for 10 min, followed by 10,000 g for 25 min and then 100,000 g for 60 min. The resulting cytosol fraction (250 ml) was applied to a 5-ml SMTP-column pre-equilibrated with 76 mM sodium phosphate, pH 7.4, containing 0.1 mM EDTA (buffer B). After washing with buffer B (200 ml), the column was developed with 60 ml of buffer B containing 10 µM 12-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA). The eluate was dialyzed against buffer B to remove AUDA and ultrafiltered to concentrate and exchange buffer to 100 mM sodium phosphate, pH 7.4, containing 3 mM dithiothreitol. From 3 batches of affinity chromatography, 2.2 mg of homogeneous sEH was purified. The purified sEH had specific activities of 511 nmol min⁻¹ mg⁻¹ for the Cterm-EH and 2,850 nmol min⁻¹ mg⁻¹ for the Nterm-phos when we used trans-stilbene oxide and p-nitrophenyl phosphate as respective substrates.

LC-MS Analysis of EET and DHET

Samples to be analyzed were extracted with ethyl acetate. After centrifugation,
supernatant was concentrated to dryness. The resulting materials were dissolved in methanol and subjected to LC-MS analysis for 14,15-EET and 14,15-DHET on a Micromass Quattro Ultima triple quadrupole tandem mass spectrometer equipped with an electrospray ionization (ESI) interface (Waters, Tokyo, Japan). Samples (10 µl) were resolved on a silica-ODS column (100 × 2 mm; Pegasil ODS SP100-3, Senshu Scientific, Tokyo, Japan) developed at 0.2 ml min\(^{-1}\) with a linear gradient of acetonitrile in 0.1% formic acid (60–100%) for 15 min. The ESI was performed in the negative ion mode with a capillary voltage at 3.0 kV. The cone voltages were set at 35 V for 14,15-DHET/14,15-DHET-\(d_{11}\) and 30 V for 14,15-EET/14,15-EET-\(d_{11}\). Data was acquired in the multichannel analysis mode and analyzed using the MassLynx software (Ver. 3.5; Waters).

**Assay for sEH**

The Cterm-EH activity was assayed with 14,15-EET or (3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester (PHOME) as a substrate. When using PHOME, we preincubated mouse sEH (60 ng) for 10 min in 80 µl of 25 mM Bis-Tris-HCl, pH 7.0, containing 0.1 mM MgCl\(_2\) and 0.1 mg ml\(^{-1}\) bovine serum albumin (buffer C) with or without a compound to be tested. The composition of buffer C was based on the method by Tran et al. (98), unless MgCl\(_2\) was added to unify the buffer composition with that for Nterm-phos determination, in which MgCl\(_2\) is essential. After adding 20 µl of the substrate, fluorescence (excitation, 355 nm; emission, 460 nm) of the reaction product was measured kinetically at 30°C. The final concentrations of sEH and PHOME in the standard assay conditions were 4.7 nM and 12.5 µM, respectively. When using EET, we incubated mouse sEH (0.18 ng) with 14,15-EET in 60 µl of buffer C at 30°C for 20 min. After addition of 14,15-EET-\(d_{11}\) and 14,15-DHET-\(d_{11}\) (25 pmol and 5 pmol, respectively) as internal standards, 14,15-DHET formed was determined by LC-MS as described above. The Nterm-phos activity was assayed with AttoPhos as a substrate. Mouse sEH (30 ng) was preincubated for 10 min in 80 µl of buffer C with or without a compound to be tested. After adding 20 µl of AttoPhos, fluorescence (excitation, 450 nm; emission, 545 nm) of the reaction product was measured kinetically at 30°C. The final concentrations of sEH and AttoPhos in the standard assay conditions were 2.3 nM and 5 µM, respectively.

**Assay for 14, 15-EET Hydrolysis in Cultured Cells**

HepG2 cells (5 × 10\(^5\) cells) were seeded on 24-well plates and cultured overnight. Cells were then washed with Hanks’ balanced salt solution containing 20 mM Hapes, pH 7.4, 0.1 mg ml\(^{-1}\) bovine serum albumin, and 1 mM MgCl\(_2\) (buffer D) and subsequently treated with
various concentrations of SMTP-7 (0-30 µM) in 500 µl of buffer D for 10 min. After addition of 14, 15-EET (0.3 µM) to the culture for 40 min, the reaction was stopped by adding 2-propanol (300 µl). After addition of 14,15-EET-d_{11} and 14,15-DHET-d_{11} (200 pmol and 10 pmol, respectively), the amounts of 14,15-EET remaining and 14,15-DHET formed were determined by LC-MS as described above.

**Global Analysis of Arachidonate Metabolites of the Cytochrome P450, Cyclooxygenase, and Lipoxygenase Pathways**

Plasma (400 µl) obtained from Guillain-Barré syndrome model rats 2 h after SMTP-7 or saline treatment (n = 8) on day 13 was randomly paired within each group, and the mixture (n = 4 for each group) was centrifuged at 5,000 g for 10 min. The resulting supernatant was mixed with 80 µl formic acid, and the mixture was applied to Sep-Pak C18 Plus Short Cartridges (Waters, Tokyo, Japan). The column was washed with EtOH-water-formic acid (10:100:1, vol⁻¹ vol⁻¹), and metabolites of interest were eluted with 5 ml EtOH. The eluate was evaporated and dissolved with 40 µl of 50% aqueous MeOH. Aliquots (10 µl) were subjected to LC-MS/MS analysis on an L-column2 ODS (2 µm, 1 × 150 mm, CERI, Tokyo, Japan) developed at a rate of 0.1 ml min⁻¹ with a liner gradient (10–85%) of acetonitrile in 5 mM ammonium formate-formic acid (1000:1, vol⁻¹ vol⁻¹) for 26 min. Eluates were ionized with ESI, and negative ions of oxylipins were monitored on API 3200 QTRAP (AB SCIEX, Tokyo, Japan). Metabolites to be analyzed were the following 48: (±)-5,6-DHET, (±)-8,9-DHET, (±)-11,12-DHET, (±)-14,15-DHET, (±)-5,6-EET, (±)-8,9-EET, (±)-11,12-EET, (±)-14,15-EET, prostaglandin (PG)A2, PGB2, PGD2, 6-keto-PGE1, PGE2, 6-keto-PGF1α, PGF2α, 15-keto-PGF2α, 2,3-dinor-8-iso-PGF2α, PGJ2, δ-12-PGJ2, 15-deoxy-δ-12,14-PGJ2, thromboxane (TX)B2, 11-dehydro-TXB2, leukotriene (LT)B4, 12-keto-LTB4, 20-COOH-LTB4, LTC4, LTD4, LTE4, LTF4, 20-OH-LTB4, 5(S)-hydroxyeicosatetraenoic acid (HETE), 8(R)-HETE, 9(S)-HETE, 11(R)-HETE, 12(R)-HETE, 15(S)-HETE, 16(R)-HETE, 19(S)-HETE, 20-HETE, 5(S)-hydroperoxyeicosatetraenoic acid (HPETE), 12(S)-HPETE, 15(S)-HPETE, 5-oxo-eicosatetraenoic acid (ETE), 12-oxoETE, 15-oxoETE, 5(S),6(S)-lipoxin (LX)A4, 5(S),14(R)-LXB4, and hepoxilin A3.

**Assay for plasminogen activation**

The activation of plasminogen was assayed by measuring initial velocity for urokinase-type plasminogen activator-catalyzed plasmin generation using the chromogenic substrate H-Val-Leu-Lys-p-nitroanilide. A reaction mixture consisting of 50 nM plasminogen, 50 U ml⁻¹ urokinase-type plasminogen activator and 0.1 mM H-Val-Leu-Lys-p-nitroanilide in
50ml of buffer (50 mM Tris-HCl, 100 mM NaCl and 0.01% Tween 80, pH 7.4) was incubated in the presence or absence of SMTP congeners at 37°C. The hydrolysis of H-Val-Leu-Lys-\(p\)-nitroanilide (absorbance at 405 nm) was kinetically monitored for up to 60 min. From the slope of the plots of \(A_{405}\) versus \(t^2\), the initial velocity of plasmin generation was calculated.
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And finally, I profoundly hope the successful development of SMTPs as a novel, excellent stroke drug.
References


Supplementary Information for

The analysis of the anti-inflammatory mechanism of the novel stroke drug SMTP

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United Graduate School of Agricultural Science
Department of Applied Life Science
MATSUMOTO Naoki

Supplementary Table 1. Results of the peptide mass fingerprinting analysis of protein band 1 in Fig. 2.2B
Supplementary Table 2. Activity of SMTPs against plasminogen, Cterm-EH, and Nterm-phos.
Supplementary Table 3. Physicochemical properties of SMTP congeners.
Supplementary Figure 1. Mass spectra of SMTP congeners.
Supplementary Figure 2. UV spectra of SMTP congeners.
Supplementary Figure 3. IR spectra of SMTP congeners.
Supplementary Figure 4. $^1$HNMR spectra of SMTP congeners.
Supplementary Figure 5. $^{13}$CNMR spectra of SMTP congeners.
## Supplementary Table 1. Results of the peptide mass fingerprinting analysis of protein band 1 in Fig. 2.2B.

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Supplementary Table 2. Activity of SMTPs against plasminogen, Cterm-EH, and Nterm-phos.

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<th>Plasminogen modulation (-fold μM⁻¹)</th>
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<th>Nterm-phos IC₅₀ (μM)</th>
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## Supplementary Table 3. Physicochemical properties of SMTP-52, -57, -58, -60, -61, and -62.

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<th>SMTP-60</th>
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<td>C₂₉H₴₁NO₄</td>
<td>C₃₀H₴₂NO₅</td>
<td>C₃₀H₴¹NO₆</td>
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<td>499.2464 (M – H)^−</td>
<td>450.2630 (M + Na)^+</td>
<td>468.3136 (M + H)^+</td>
<td>482.3261 (M + H)^+</td>
<td>512.3009 (M + H)^+</td>
<td>484.3072 (M + H)^+</td>
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<td>450.2615 for C₂₉H₴₂NO₄</td>
<td>468.3080 for C₂₉H₴₁NO₄</td>
<td>482.3265 for C₃₀H₴₂NO₅</td>
<td>512.3007 for C₃₀H₴¹NO₆</td>
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<td>UV λmax nm (c) MeOH</td>
<td>215 (39,200), 259 (8,800), 292 (2,800)</td>
<td>210 (36,600), 259 (5,500), 299 (1,900)</td>
<td>212 (40,600), 259 (5,900), 302 (2,480)</td>
<td>216 (35,800), 258 (7,700), 302 (3,800)</td>
<td>216 (53,400), 259 (12,100), 302 (3,800)</td>
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### 'H NMR

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<td>Molecular formula</td>
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<td>C₂₉H₴₂NO₄</td>
<td>C₂₉H₴₁NO₄</td>
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<td>MS m/z Found</td>
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<td>482.3261 (M + H)^+</td>
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<td>450.2615 for C₂₉H₴₂NO₄</td>
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<td>−35.1° (c 0.73)</td>
<td>−30.9° (c 0.50)</td>
<td>−13.7° (c 0.50)</td>
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<tr>
<td>SMTP-62</td>
<td>−35.1° (c 0.73)</td>
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<td>−13.7° (c 0.50)</td>
<td>−14.1° (c 1.0)</td>
<td>−12.7° (c 1.0)</td>
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* Methanol-d₄ for SMTP-52 and acetone-d₄ for others at 400 MHz for 'H and 100 MHz for 'C (for SMTP-57) or at 600 MHz for 'H and 150 MHz for 'C (for others).
Supplementary Figure 1A. Mass spectra of SMTP congeners. (SMTP-52, negative ion mode)
Supplementary Figure 1B. (SMTP-57, positive ion mode)
Supplementary Figure 1C. (SMTP-58, positive ion mode)
Supplementary Figure 1D. (SMTP-60, positive ion mode)
Supplementary Figure 1E. (SMTP-61, positive ion mode)
Supplementary Figure 1F. (SMTP-62, positive ion mode)
Supplementary Figure 2A. UV spectra of SMTP congeners. (SMTP-52)

B. (SMTP-57)
Supplementary Figure 2C. (SMTP-58)

D. (SMTP-60)
Supplementary Figure 2E. (SMTP-61)

F. (SMTP-62)
Supplementary Figure 3A. IR spectra of SMTP congeners. (SMTP-52)

B. (SMTP-57)
Supplementary Figure 3C. (SMTP-58)

D. (SMTP-60)
Supplementary Figure 3E. (SMTP-61)

F. (SMTP-62)
Supplementary Figure 4A. $^1$HNMR spectra of SMTP congeners. (SMTP-52)
Supplementary Figure 4B. (SMTP-57)
Supplementary Figure 4E. (SMTP-61)
Supplementary Figure 5A. $^{13}$C NMR spectra of SMTP congeners. (SMTP-52)
Supplementary Figure 5B. (SMTP-57)
Supplementary Figure 5C. (SMTP-58)
Supplementary Figure 5D. (SMTP-60)
Supplementary Figure 5E. (SMTP-61)
Supplementary Figure 5F. (SMTP-62)