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Targeting endothelin receptors for pharmacotherapy of ischemic stroke: current scenario and future perspectives

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Increased expression of endothelin (ET) peptide and its receptors following ischemic stroke is found to regulate many critical aspects of stroke pathophysiology. Many attempts have been made to target ET receptors in various animal models of stroke, but it is very difficult to draw a definite line of conclusion, because these studies differ in many aspects, such as animal model, treatment schedule, parameters and techniques used for assessing these parameters. A meta-analysis of all studies showed a significant reduction in the lesion volume and improvement in functional outcome in focal cerebral ischemia. ET_A receptor antagonists appear to offer an essential advantage of multiple neuroprotective mechanisms, including prevention of blood–brain barrier disruption and leukocyte infiltration.

Stroke is an acute neurological injury occurring as a result of interruption of blood supply to a part of the brain, typically caused by a thrombus occlusion or embolus or hemorrhage owing to rupture of blood vessels. Stroke is the third leading cause of death and first major cause of the long-term disability and has a huge socioeconomic impact, worldwide [1].

The pathophysiology of stroke is extremely complex and involves highly interlinked cascades of several pathogenic pathways involving a myriad of mediators [2]. Many molecular targets have been tried to achieve therapeutic benefit in stroke [2–10]. Despite the extensive research going on in this area, there is still unavailability of clinically effective neuroprotective agents to ameliorate the deleterious effects of stroke. Hence there is a need for identifying new therapeutics target for stroke treatment.

Endothelin (ET) is one of the most potent vasoregulator known to man and has a crucial role in post-ischemic hypoperfusion. Beyond being a potent vasoconstrictor, ET is thought to modulate many crucial aspects of stroke pathogenesis [11]. Therapeutic potential of ET antagonists in stroke has been suggested by many researchers [11,12]. In this article we focus on ET as a potential therapeutic target in ischemic stroke. A detailed account of ET system in connection with stroke pathophysiology has been presented and various preclinical attempts targeting ET system in ischemic stroke have been discussed.

Endothelin

ET was discovered in 1985 as endothelium-derived contracting factor (EDCF) by Hickey *et al.* [13] and subsequently, Yanagisawa and co-workers named it 'Endothelin' [14]. ETs are a family of peptides consisting of three members namely ET-1, ET-2 and ET-3. They have 21 amino acid open loop tertiary structures, formed because of two disulphide bonds joining the two cysteine amino acids. These peptides are encoded by different genes known as preproendothelin genes. These genes are translated into respective 212 amino acid long peptides known as preproendothelins (preproET). The preproET is cleaved in the cytoplasm by neuronal endopeptidase furin, forming a biologically inactive precursor 'big ET' which have 37–41 amino acid residues. The big ET is then acted upon by endothelin converting enzymes (ECE) to form active 21 amino acid peptide and inactive C terminal fragment (Fig. 1). All the ET isoforms share a remarkable structural similarity in having a 21 amino acid structure, two disulphide bonds and hydrophobic C

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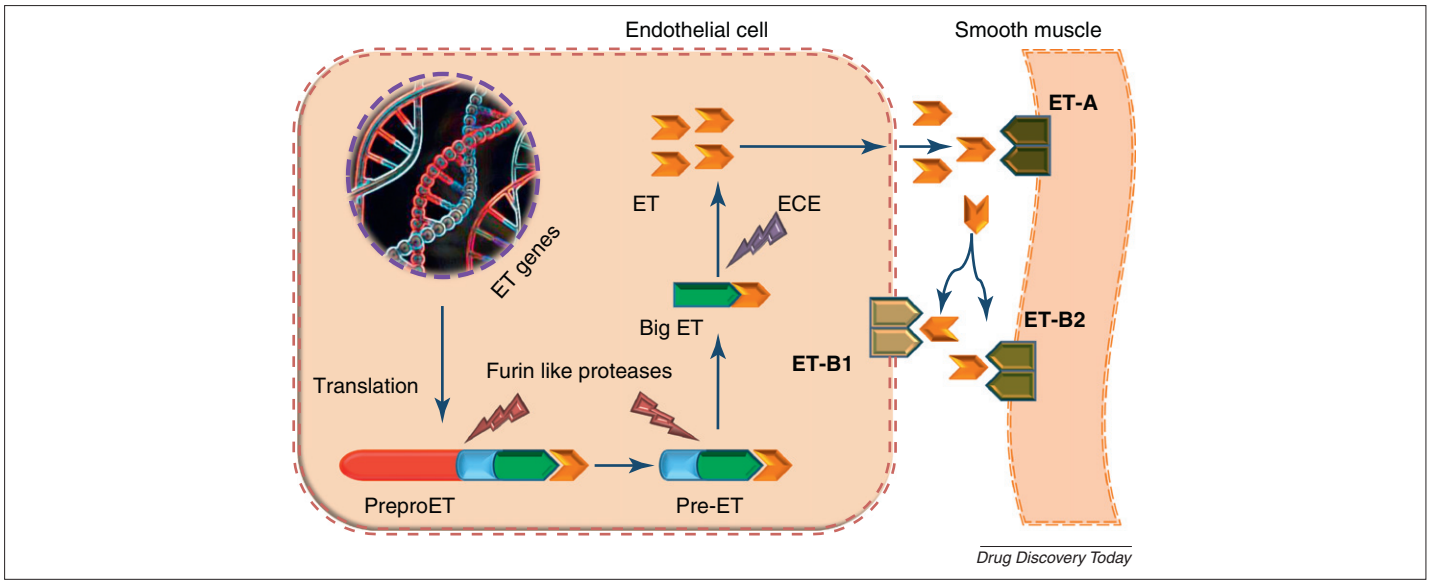

FIGURE 1

Biosynthesis of ET peptide. ET synthesis begins with the transcription of the preproET gene. PreproET mRNA translation results in the formation preproET, which is cleaved at dibasic sites by a furin-like endopeptidase, to form big ET-1, -2, and -3. Big ET is cleaved by ECE, metalloprotease and chymase to produce ET isoforms. These endothelins act on ET receptors ET_A and ET_B present on smooth muscles and endothelium respectively to produce the downstream actions. *Abbreviations:* ECE: endothelin converting enzymes; ET: endothelin.

terminal hexapeptide structure [15]. ET-2 and ET-3 differ from ET-1 in two and six amino acid residues, respectively. Among the ET isoforms, ET-1 is probably the only member released from endothelium. The other isoform namely ET-2 is produced by the small intestine and kidney whereas ET-3 is found in high quantities in intestine and nervous tissue [16].

Endothelin receptors

ET receptors are G-protein-coupled receptors belong to the rhodopsin family but they have a very long extracellular N-terminal. The receptor consists of seven transmembrane (TM) stretches of 20–27 hydrophobic amino acids. The TM-4 and TM-6 domains are specifically meant for ligand interaction. ET receptors are mainly classified as ET_A and ET_B and have 59% sequence homology. ET_B is further classified as ET_{B1} and ET_{B2}. ET_A receptors are present predominantly on vascular smooth muscle whereas ET_B receptors are abundantly present on endothelium and vascular smooth muscles [17,18]. A brief account of the ET receptor is presented in Table 1.

Transmembrane signaling

Activation of phospholipase C (PLC) pathway leading to diacylglycerol (DAG) and D-myo-inositol-1,4,5-trisphosphate (IP3) is a central theme of ET mediated transmembrane signaling. This pathway leads to a rise in intracellular calcium (Fig. 2). Rise in intracellular calcium through ET_A and ET_B receptors located on smooth muscle leads to vasoconstriction. By contrast, ET_B receptor activation on endothelium leads to nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS). Although the link between ET_B and eNOS activation is not clear, the elevation of intracellular calcium and protein kinase B and/or Akt phosphorylation might have a role in activation of eNOS [19,20].

Endothelin system in the central nervous system

There is ample evidence indicating a wide distribution of components of the ET system in the central nervous system (CNS) [21–23]. Data from histological studies of human brain indicated that there is widespread distribution of all the components of ET system. These data are in agreement with localization studies reported in animals [24].

ET-1 has been found to be localized in approximately 24 regions of the human brain, including diencephalon, brainstem, basal nuclei, cerebral cortex, cerebellar hemisphere, amygdala, pineal gland, post central gyrus and hippocampus. ET-1 mRNA is found to be co-localized with ET-1 in all these regions except for pineal

TABLE 1

A brief account on the ET receptors, their signaling component and ligands

Receptor	Amino acids	Location on chromosome	Gene	Transducer	Effectors	Endogenous ligand selectivity	Selective agonists	Antagonists	
								Selective	Mixed
ET_A	427	4q31.22	EDNRA	G_q/G_{11}	PLC, PLA_2 and PLD	ET-1 = ET-2 ≫ ET-3	None	PD156707, S-0139, SB234551, BQ610, A127722, BQ123 BSF208075	SB217242 Bosentan TAK-044
ET_B	442	13q22	EDNRB	G_s, G_i/G_0 and G_q/G_{11}	PLC, PLA_2 and PLD	ET-1 = ET-2 = ET-3	Sarafotoxin, IRL-1620, BQ3020	BQ788, IRL-2500, A192621, IRL-2500, IRL-1038	SB209670

EDNRA: endothelin receptor type A genes, EDNRB: endothelin receptor type B genes, PLA_2: phospholipase A2, PLD: phospholipase D.

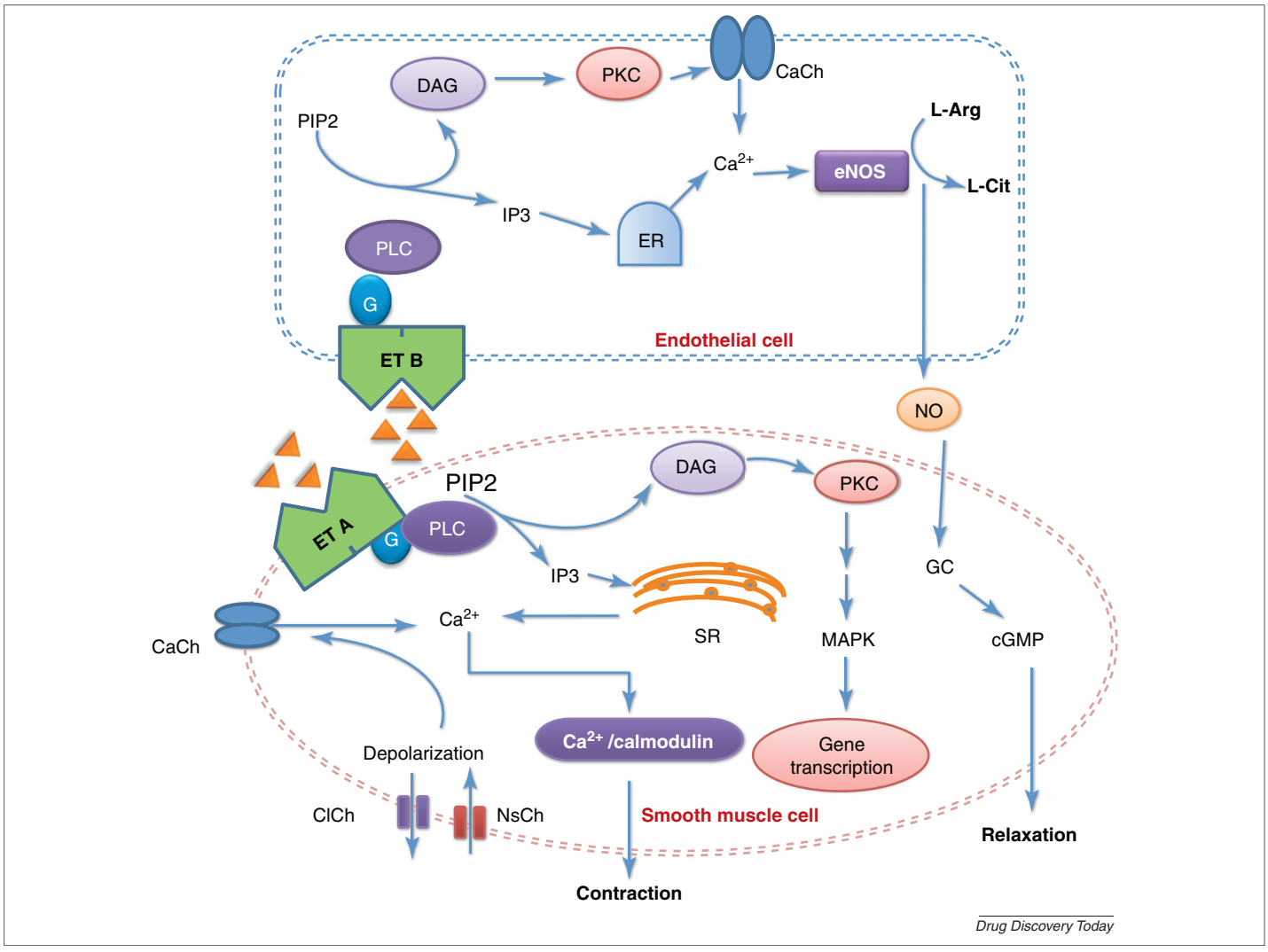

FIGURE 2

Transmembrane signaling of ET receptors. ET receptor activation leads to diverse cellular responses by regulating the intracellular calcium. Activation of PLC cleaves PIP2 to form DAG and D-myo-inositol-1,4,5-trisphosphate (IP3). IP3 activates IP3 receptor on the ER and regulate the Ca^{2+} release from the ER stores. The ET_A receptor activation also opens the endothelin linked CaCh. ETs have also been shown to induce cellular depolarization via influx of Na^+ by NsCh and efflux of Cl^- by ClCh. This increase in intracellular calcium is responsible for contraction. DAG further activates PKC which in turn regulates gene transcription by MAPK signaling. Stimulation of endothelial ET_B receptors activates signaling pathways that promote the release of relaxing factors such NO. NO results in vaso-relaxation by increasing 3′,5′-cyclic guanosine monophosphate (cGMP) production through GC. *Abbreviations:* CaCh: Ca^{21} channel; cGMP: cyclic guanosine monophosphate; ClCh: chloride channels; DAG: diacylglycerol; ER: endoplasmic reticulum; ET: endothelin; GC: guanylate cyclase; IP3: inositol-1,4,5-trisphosphate; MAPK: mitogen-activated protein (MAP) kinases; NO: nitric oxide; NsCh: nonselective cation channels; PLC: phospholipase C; PKC: protein kinase C; PIP2: phosphatidyl inositol 4,5-bisphosphate; SR: Sarcoplasmic reticulum.

gland and post central gyrus [25]. ET converting enzyme-1 (ECE-1) immunoreactivity has been found in endothelial cells of cerebral vasculature and also in neurons. Presence of ECE-1 and ET-1 mRNA confirms local synthesis of ET-1. Former has been found in nine regions of human brain namely vascular smooth muscles, choroidal epithelial cells, neurons in the diencephalon, hippocampus, amygdala, dentate nucleus, Purkinje cells of the cerebellum, flocculo-nodular lobe and vermis whereas the latter is more widely distributed [26]. Moreover systemically administered radiolabeled ET in rat was found to bind to choroid plexus, median eminence and the subfornical region [which together constitute the blood–brain barrier (BBB)], which indicate that ET does not cross the BBB [27]. This locally synthesized ET-1 is thought to mediate its effects through ET_A and ET_B receptors which indicate that ET does not cross the BBB and it is synthesized locally.

ET receptors possess some of the highest reported protein densities in the brain, up to 5000 fmol/mg of protein. The density of ET receptor is found to decrease from hindbrain to forebrain. Distribution is sparse in hypothalamus and cortex as compared with the brain stem, cerebellum and hippocampus [28,26]. In addition, there is evidence that ET receptors are also present in vascular smooth muscle cells, neurons, endothelial cells and astrocytes [29]. None of these receptors have been localized in glial cells in normal human brain. It is worth noting that ischemic injury caused marked expression of ET_B receptors in glial cells [30]. Strong presence of ET system in neurovascular unit, namely capillary endothelial cells and epithelial cells of the human choroid plexus, indicate their probable role in the regulation of BBB and cerebrospinal fluid (CSF) [26]. Thus, the widespread expression and pattern of ET component in brain is an indicative of its immense

importance in the physiological and pathological processes of brain.

Role of endothelin in stroke pathophysiology

The vasoconstrictive property of ET provides a logical support for its involvement in stroke. Its presence and contribution to the pathophysiological mechanisms underlying stroke is deemed to be certain, but the extent to which it contributes and the manner in which it affects the outcome of ischemic insult, is debatable and is of particular interest for the researchers to design new therapeutic strategies for stroke treatment. In this section we have made an attempt to delineate the role of ET system based on the experimental evidence available in the literature.

Elevated levels of endothelin after stroke: temporal and spatial variations

ET levels in ischemic stroke can be studied with respect to the time period (early and late) and location (plasma and CSF) of their measurement. It is interesting to note that plasma ET levels are found to fluctuate even in normal (healthy) individuals [31] and a similar fluctuation of ET levels was also seen in stroke patients. Ziv *et al.* reported almost fourfold rise in plasma ET levels of patients during the first 24 hours of stroke [32], but when plasma ET levels were measured over prolonged periods (1 week, 1 month and 3 months after stroke) they were found to be in normal range [33]. In a rat model of ischemic stroke, Matsuo *et al.* pointed out that brain ET-1 level did not change during the first three hours, increased at six hours, and remain elevated until 48 hours in ischemic as well as in penumbral region after focal cerebral ischemia in rat [34]. This is indicated by ET-1 mRNA levels which reach its maximum at 24 hours after ischemia, whereas the contractile response of the cerebral blood vessels demonstrates its greatest intensity at 48 hours [35]. This lag time of 24 hours is the time for ET-1 peptide transcription from mRNA. Thus it can be concluded that ET levels in plasma in addition to in brain tissue are increased during the early phase of ischemic stroke.

ET levels in brain and CSF are more likely to be elevated than those in plasma. This is because, ET is almost invariably released towards abluminal side (toward blood-vessel wall) and not into the lumen of the blood vessel. Hence, it is important to consider CSF or tissue concentrations of ET after ischemic onset. Barone *et al.* were the first to demonstrate the increased tissue content of ET-1 24 hours after focal in addition to global ischemia in rats, using both permanent middle cerebral artery occlusion (MCAO) and transient MCAO [36]. Lampl *et al.* observed elevation of ET in the CSF of ischemic rats [37]. In another study carried out in rabbits, elevated ET levels were observed in brain tissue as well as in plasma [38]. This suggests the autocrine–paracrine activities of ET and also establishes ET as a local mediator. The extent of ET levels depends on the duration and intensity of ischemic insult [39]. Recently, activated microglias have been shown to be prominent source of elevated ET-1 in post-ischemic brain [40]. It can be summarized that ET levels are increased in plasma as well as in brain after ischemic onset, although they are more likely to be elevated in brain tissue. The time period of this elevation can vary depending upon the intensity and duration of ischemic insult and subsequent reperfusion injury.

Correlation between infarct volume and endothelin levels

Elevated ET levels have been shown to be correlated with the degree of ischemic brain injury. The size of infarct has a close association with plasma ET levels and is expected to increase with elevated ET concentrations. Franceschini *et al.* have observed that mean 24 hours plasma ET-1 levels correlated well with mean size of the lesion in stroke patients [41]. Over expression of ET-1 is known to exacerbate ischemic damage [42] whereas ET antagonists were found to attenuate its outcome [12,43,44]. These studies also suggest that raised ET levels contribute significantly to observed infarct volume.

Receptor upregulation in post-ischemic brain

First evidence of altered vascular reactivity of ET receptors after cerebral ischemia was shown in the study conducted by Touzani *et al.* [45]. One of the important observations of the study was that the ability of BQ-3020 (selective ET_B receptor agonist) to elicit dilation was lost within 30 min of induced focal ischemia in cat [45]. Later on it was revealed that ET_A and ET_B receptors of middle cerebral artery are upregulated in post-ischemic rat brain [46]. Surprisingly, the phenomenon of ET_B receptor upregulation after stroke is observed even in organ culture of MCA [47]. Also the entire ipsilateral cortex showed the increased expression of ET receptors. ET_B receptor immunoreactivity was observed in activated microglia and/or macrophages, beginning 24 hours after MCAO [48]. This lead to the hypothesis that the increased expression of ET_B in neurons, glia and macrophages could be related to a more generalized activation of survival mechanisms, involving elements of the neurovascular unit [30]. It is important to note that the brain is heterogeneous tissue and changes in receptor expression might not be observed uniformly throughout the brain. Therefore, the differential nature of ET receptor expression needs to be understood. Although vascular ET_A receptors are upregulated, ET_A receptors at mossy fiber terminals are downregulated after ischemia leading to increased glutamate secretion by these neurons and subsequently causing excitotoxicity [30]. This heterogeneity in the receptor expression might be the reason why Chuquet *et al.* reported no change in the ETB mRNA in acute as well as chronic stages of cerebral ischemia [49].

An interesting study further demonstrated a marked (approximately 45%) downregulation of ET_A receptors brains of $ET_B^{-/-}$ knockout mice which indicates that ET_A receptor expression is in some way dependent on ET_B [50]. Researchers also made an attempt to unveil intracellular mechanisms of receptor upregulation and protein kinase C (PKC) and ERK1/2 mitogen-activated protein (MAP) kinase (MAPK) pathways were suggested to have an important role in the expression regulation [47].

It can be concluded that after ischemic stroke ET receptors are upregulated specifically in MCA and in activated microglia. Receptor upregulation in other regions of the brain might not be observed uniformly.

Impact of elevated ET and upregulated ET receptors

Although the evidences for direct neurotoxic effects of ET are rare, the elevated ET levels and its receptor expression significantly affect the outcome of ischemic brain damage. The possible mechanisms of ET mediated neuronal damage after ischemic stroke are illustrated in following subsections.

Endothelin mediated delayed hypoperfusion and excitotoxicity

ET peptides are known to cause contraction of cerebral arteries in a dose-dependent manner [51]. Overexpression of vasoconstrictive ET peptides after stroke may cause severe hypoperfusion. Pierre *et al*. established the importance of ET_A receptors in ET-1-induced constriction of human pial arteries and suggested that ET_A receptor antagonists might provide dilatory benefit in cerebrovascular disorders associated with raised ET levels [52]. Consistent with this hypothesis, many ET receptor antagonists have counteracted delayed hypoperfusion following ischemic onset [43,53,54].

ET evokes the excitatory neurotransmitter release in rat brain in a concentration-dependent manner and this is reflected in infarct volume [55]. The responses of neuronal cells to these raised neurotransmitter levels are also augmented by ET [56]. These findings support the hypothesis of the involvement of N-methyl-D-aspartate (NMDA) receptors in ET mediated neurotoxicity which was based on the finding that a NMDA antagonist improved the striatal dysfunction caused by ET exposure at a much lower concentration than that required for NMDA antagonism [57].

There can be two different mechanisms operative behind ET mediated glutamate release and subsequent excitotoxicity. One prominent mechanism is local ischemia as a result of profound vasoconstriction caused by ET. Reduced blood supply leads to energy depletion and failure of ion pumps, which maintain ionic gradients across the membrane. The resultant depolarization opens voltage-dependent calcium channels prompting glutamate release [55]. A second mechanism behind ET mediated glutamate release is independent of its vasoconstrictive actions. ET promotes glutamate release from cultured astrocytes, with the ET_B receptor having a prominent role [58]. The uptake of extracellular glutamate by glial cells is also inhibited by ET through PKC dependent mechanisms [59]. Inhibition of glutamate transporter at transcription level is thought to be behind reduced glutamate uptake [60].

Disruption of the BBB and edema formation

ET-1 being a strong vasoconstrictor might alter the capillary surface area resulting in a leaky vasculature. It is also known to alter the permeability of human cerebromicrovascular endothelial cells [61]. Several studies have suggested that ET might contribute to post-ischemic BBB damage and elevated ET-1 serum levels were found to be coupled with severe brain edema in acute stroke patients [62]. Edema in ischemic brain was attenuated by S-0139, an ET_A antagonist [34]. ET not only contributes to vasogenic brain edema by BBB damage but also results in cytogenic edema. ET mediated post-ischemic hypoperfusion further adds to the energy crisis and subsequent ionic failure in brain. This results in accumulation of ions inside the cell culminating into cytogenic edema [11]. Kawai *et al*. have demonstrated that ETs stimulated sodium uptake in rat brain endothelial cells through ET_A which suggests the role of ETs in regulating ion transport across the BBB [63]. Accumulation of Na^+ ions pulls water inside the cells resulting in edema formation, which initiates a vicious cycle. Edema compresses the brain vasculature, as brain is enclosed in a solid vault, often causing more severe brain ischemia.

Endothelin as a facilitator in post-ischemic neuroinflammation

ET has also been speculated to have a proinflammatory role in the brain [64]. ET mediated disruption of BBB paves a path for the entry of inflammatory cells in the brain. ET-1 promotes leukocyte adhesion through intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). ET-1 can promote leukocyte endothelium interactions by increasing the expression of E-selectin on endothelium [65]. ET_A antagonist, BSF-208075 has been shown to reduce the post-ischemic leukocyte activation in ischemic brains [66]. Besides its role on inflammatory cell recruitment, ET also influences the release of certain key inflammatory mediators which are known to be involved in ischemic brain damage. Human CNS-derived endothelial cells when exposed to ET-1 secrete interleukin-8 [67]. ET-1 also induces the release of arachidonic acid by stimulating phospholipase A2 (PLA_2) [68]. In macrophages, ET-1 increases cyclooxygenase-2 expression and prostaglandin-E2 production [69]. These evidences highlight the proinflammatory role of endothelins after cerebral ischemia.

Strategies targeting endothelin system in stroke

The key components of ET system that can be targeted to ameliorate the outcome of cerebral ischemic reperfusion (IR) injury are discussed below.

ECE

ECE converts ET precursor 'big endothelin' to a 21 amino acid functional peptide ET-1, which is known to worsen the outcome of ischemic brain injury. Phosphoramidon, a nonselective ECE inhibitor has been shown to attenuate the ischemic brain damage in rats [70]. SLV 338 is another non-specific inhibitor of ECE that improved survival chances in stroke prone spontaneously hypertensive rats [71]. Melatonin has also been shown to exhibit beneficial effects in cerebral ischemia by inhibiting ECE [72]. All the above-mentioned ECE inhibitors are highly nonspecific and studies demonstrating therapeutic potential of selective ECE inhibitors will be more useful to validate ECE as a therapeutic target. Neuroprotective effect of CGS263O3, a selective ECE inhibitor, in cerebral ischemia has further suggested that targeting ECE could be a useful therapeutic strategy to ameliorate post-ischemic brain damage [73], However, there are evidences indicating non-ECE dependent pathways into the formation of ET-1 from big ET [74]. This is consistent with the fact that transgenic mice lacking ECE-1 and ECE-2 were also shown to produce ET-1 [75]. These evidences suspect utility of ECE as a target and further studies are warranted to validate ECE as a therapeutic target for stroke treatment.

Endothelin receptors

ET exerts most of its deleterious effects by acting on its receptors and hence ET receptors might serve as the best therapeutic target in ET system.

Possible strategies targeting ET receptors can be:

(i) Selective antagonism of ET_A receptors.
(ii) Dual antagonism of ET_A and ET_B receptors.
(iii) ET_B receptor agonists.

Utilizing first strategy many studies has indicated the beneficial effects of selective ET_A antagonists in ischemic stroke and these studies have been discussed in the next section. Researchers have also demonstrated the neuroprotective potential of dual ET antagonists in cerebral ischemia [76–78] but bosentan, a dual acting antagonist, failed to show neuroprotection in MCAO model in rats [70]. Therefore neuroprotective potential of dual antagonist

is a matter of debate. ET_B receptors on endothelium mediate vasodilatation by releasing endothelium derived relaxing factor (EDRF), prostacyclin and selective ET_B blockade causes paradoxical constriction of basilar arteries [79]. Moreover, ET_B receptors also act as clearance receptors for ET-1 [80] and ET_B receptor blockade has been found to exacerbate ischemic brain damage indicating that ET_B receptors are vasculoprotective [49]. Recently, IRL-1620, a highly selective ET_B agonist, treatment has shown significant neuroprotection in permanent focal cerebral ischemia in rats. IRL-1620 treated ischemic animals showed significant reduction in brain injury and a marked improvement in the neurological and motor function tests [81]. Thus selective ET_B receptor agonism seems like a logical therapeutic strategy in stroke treatment.

Endothelin receptor antagonists: do they really work?

The development of specific ET receptor antagonists began in the early 1990s.

Therapeutic approach of utilizing ET receptor antagonist in cerebral ischemia was initially sought on the basis of the following observations [70]:

(i) Cultured endothelial cells release ET-1 in response to stress, hypoxia and injury.
(ii) Exogenously administered ET-1 in brain can reduce the CBF below threshold and cause ischemic damage.
(iii) Brain ET-1 levels are increased following ischemia.

In later studies, researchers focused on ET-1 mediated neuronal damage, its proinflammatory role and contribution to post-ischemic hypoperfusion. There has been increased understanding of the delayed secondary injury occurring after ischemic or hemorrhagic onset. ET mediated cerebral vasospasm is the principal factor behind this injury. Therefore many ET receptor antagonists have been developed to counteract the delayed hypoperfusion following traumatic brain injury. Effect of ET and ET receptor antagonist on hemodynamics of post-traumatic brain has been reviewed [82].

Several research groups have evaluated the therapeutic potential of ET receptor antagonists in a variety of experimental models using different animal species and parameters (Tables 2 and 3). They came up with confounding results and hence it is difficult to draw a definite line of conclusion from these studies.

Reduction in the infarct volume or cell death is considered as a gold standard to evaluate neuroprotective potential of interventions in cerebral ischemia. Parameter of increase in the number of surviving neurons can be considered as an equivalent to infarct volume.

In models of global cerebral ischemia in gerbils (Table 3), the number of surviving neurons in the cornus ammon (CA1) region can stand as a direct indication of neuroprotective potential of intervention. In one such study, cortical neurons (n/mm^2) were dose-dependently protected when treated with BSF-208075, 10 min after reperfusion [66]. Similarly, SB 209670 was found to

TABLE 2

Effects of ET receptor antagonists in animal models of focal cerebral ischemia

S.N.	Compound	Model	Treatment Details	Outcome	Ref
1	PD156707 (ETA selective)	Permanent MCAO by craniotomy in cats, sacrificed at six hours	Post-occlusion: 3 μmol/kg (i.v. bolus), 5 μmol/(kg hours) infusion, Starting from 30 min of MCAO to six hours	Restoration of CBF at six hours of MCAO and reduction in infarct volume	[1]
2	Ro 61-1790 (ETA selective)	MCAO for 90 min followed by 24 hours reperfusion in cats	Post-reperfusion: 10 mg/kg, i.v. bolus followed by the 4-mg/(kg hours) infusion for six hours beginning just at onset of reperfusion	No significant difference in infarct volume or neurologic score and increased mortality	[2]
		Permanent MCAO in SHR, sacrifice at 24 hours	Pre and post occlusion, 10 mg/kg i.v., 5 min before MCAO, five and eight hours after MCAO	Reduction in infarct volume and improvement in CBF assessed at four hours	[3]
3	SB234551 (ETA selective)	Permanent MCAO in SD rats, sacrificed at 24 hours	Post-occlusion: 7.5, 15, 30 or 60 mg/kg, i.v. infusion beginning after 15 min of MCAO and continued up to six hours	Reduction in brain infarction, brain swelling and neurological deficits	[4]
		Permanent MCAO in SD rat, sacrificed at 24 hours	Pre and post occlusion, 1.8 mg/(kg hours) infusion, starting from 15 min before MCAO until 24 hours	Increase in CBF in both hemispheres by 1.7-fold	[5]
4	A-127722 (ETA selective)	MCAO for 90 min in SD rats, sacrificed at 24 hours	Post-occlusion: 5 mg/kg i.v. 30 min after MCAO and sc four hours after MCAO	Reduction in brain damage but no significant improvement in brain perfusion	[6]
5	S-0139 (ETA selective)	MCAO for one hour in Wistar rats sacrificed at 24 hours	Post-occlusion: 0.03–1 mg/(kg hours) (i.v. infusion) starting from 10 min after reperfusion continuously up to 24 hours	Reduction in infarct volume, brain edema formation, BBB disruption and mortality	[7]
6	BQ123 (ETA selective)	MCAO for two hours in SD rats followed by reperfusion, sacrificed at 24 hours	Post-occlusion: 1 mg/kg (i.v.), three time (30 min, two hours and four hours after MCAO)	Reduction in infarct volume and brain MDA content along with improvement in neurological deficit and brain GSH levels	[8]
7	SB217242 (mixed)	Permanent MCAO in SHR by craniotomy, sacrificed at 24 hours	Pretreatment: 3 or 15 mg/kg (p.o., BID) for seven days. MCAO on day 7	Reduction in infarct volume	[9]
8	TAK-044 (mixed)	MCAO in Wistar rat for two hours, sacrificed at 24 hours	Pretreatment: 5 mg/kg, i.p., for seven days	Reduction in brain damage and improvement in neurological deficits. Increase in SOD and GSH levels along with reduced lipid peroxidation	[10]
9	Bosentan (mixed)	Permanent MCAO in SD rat, sacrificed at four hours	Pre and post-occlusion: 3, 15, 30 mg/kg i.v., 15 min before or 30 min after MCAO	No improvement in CBF and neurological damage	[11]

TABLE 3

Effects of ET receptor antagonists in animal models of global cerebral ischemia

S.N.	Compound	Model	Treatment details	Outcome	Ref
1	BQ123 (ETA selective)	BCAO for 15 min, CBF monitored continuously	0.8 mg/kg (i.v.), 30 min before or 15 min after BCAO	Reversal in post ischemic hypoperfusion as well as NLA (Nω-nitro-L-arginine, NOS inhibitor) induced hypo perfusion	[12]
2	BQ610 (ETA selective)	BCAO for 15 min, sacrificed after 4 days	0.3 μmol/kg (i.v.), 15 min before BCAO	Improvement in microvascular perfusion and neurological deficits and reduction in leukocyte endothelium interactions	[13]
3	BSF-208075 (ETA selective)	BCAO for 15 min, sacrificed on day 7	5 mg/kg, 30 mg/kg (i.v.), 10 min after reperfusion	Reduction in cortical neuronal cell death and post ischemic leukocyte rolling independent of any change in cerebral blood flow	[14]
4	SB 209670 (mixed)	BCAO for 6.5 min, sacrificed on day 7	10 and 50 μg (i.c.v.), 5 min pre and 60 min post occlusion	Reduction in ischemia-induced neuronal damage	[13]

show 94% neuroprotection when administered intracerebroventricularly [83]. The exact mechanism behind this neuroprotection was not investigated. Therefore, subsequent studies concentrated on the probable mechanism of neuroprotection. Hypoperfusion following global cerebral ischemia is known to exacerbate neuronal damage. ET has been thought to be one of the contributors of post-ischemic hypoperfusion. BQ123 (selective ET_A antagonist) administered 30 min before or 15 min after bilateral carotid artery occlusion (BCAO) abolished the post-ischemic hypoperfusion [84]. The treatment also reversed the CBF changes caused by Nω-nitro-L-arginine [(NLA) the competitive inhibitor of NO synthesis]. The proinflammatory role of ET and attempts to modulate inflammatory reactions with ET antagonists has been the focus of other studies carried out in a model of global cerebral ischemia. Lehmberg *et al.* demonstrated that leukocyte endothelium interactions were reduced by the pretreatment of BQ610. There was also an improvement in microvascular perfusion (indicated by arteriovascular transit time – AVCT) and neurological score [85]. Intravital fluorescence microscopy technique used to study the leukocyte endothelium interactions in this study was also used by Hauck *et al.* They found that BSF-208075 administered 10 min after reperfusion, reduced the number of rolling leukocytes. This effect probably caused substantial neuroprotection as revealed in histopathology. Involvement of CBF in neuroprotective effect was ruled out because no significant alterations were observed [66].

The first example of ET_A antagonist showing efficacy in focal cerebral ischemia came from the study by Barone *et al.* They demonstrated that pretreatment of SB217242 for seven days reduced the infarct volume by 30% in permanent cerebral ischemia [86]. Subsequently, many studies were aimed at evaluating neuroprotective potential of ET receptor antagonism. PD155707, a ET_A receptor antagonist, when administered by intravenous infusion started after 30 min of permanent MCAO in cats not only restored the CBF but also reduced the infarct volume [87]. However in a reversible MCAO model in cat, intravenous infusion of Ro 61-1790 starting after reperfusion did not show any effect on brain damage but increased the mortality rate [88]. The infusion was done for 24 hours and hence prolonged blockade of ET_A receptors and subsequent exacerbation of brain edema could be the probable reason for the enhanced mortality.

Bosentan is the ET receptor antagonist that could not show neuroprotection in rat model of focal ischemia. The neuropathological outcome was evaluated after 4 hours of permanent MCAO and Bosentan showed no improvement in perfusion or in infarct volume [70]. It is important to note here that, Bosentan has a nonspecific action toward ET receptors. Therefore, beneficial effects of ET_A blockade could have been surpassed by ET_B blockade. Moreover, the assessment of neurological outcome was performed 4 hours after MCAO contrary to other models where assessment is done after 24 hours. With the exception of Bosentan, other ET receptor antagonists showed considerable neuroprotection in model of focal cerebral ischemia in rat. Almost all of these were selective for ET_A receptor. Extent of reduction in infarct volume varied from 25 to 40%. Because treatment schedule and model details varied among different studies, it is difficult to draw definite a conclusion from them. One important variation is the issue of treatment mode. Although prolonged pretreatment for seven days has yielded beneficial results in case of TAK-044 and SB217242, other compounds such as S-0139, SB234551 and A-127722 have shown neuroprotection upon post-treatment. Route of administration in all of these latter cases was essentially intravenous. Sometimes it was necessary to administer the ET_A antagonist as a continuous infusion (S-0139, SB234551) whereas in other cases the drug needed to be administered at multiple time points. One special case worth mentioning is the Ro 61-1790 evaluated in a model of permanent MCAO in stroke prone spontaneously hypertensive (SHR) rats. For perfusion study, a four-hour model was used whereas for a infarct volume assessment 24-hour model was used. Pretreatment with the said drug significantly improved post-ischemic CBF at four hours of MCAO as indicated by the number of fluorescent microvessels. There was significant reduction in infarct volume when the drug was administered twice following ischemia. However, this effect could not be observed with a single post-ischemic injection of the drug suggesting that adequate concentrations could not be maintained with single administration [89]. Another compound A-127722 had to be administered twice (intravenously 30 min after MCAO and subcutaneously four hours after MCAO) to maintain adequate concentration of the drug in plasma [90].

All these animal studies showed large variation in terms of experimental design, stroke model and method of assessment. To draw definite conclusions regarding efficacy of ET_A antagonists, meta-analysis of all the reported studies of focal cerebral ischemia was performed. Studies about global cerebral ischemia were

excluded because they were less in number and the assessment parameter was not uniform throughout.

Meta-analysis: efficacy of ET_A receptor antagonists in focal cerebral ischemia

Meta-analysis is a useful statistical tool when apparently conflicting data from literature is to be analyzed. The main aim of meta-analysis is to integrate the findings of different studies and provide a methodological rigor to literature review. For this purpose parameters indicating effect of treatment are defined. In current meta-analysis, lesion volume (infarct volume) was considered as a parameter indicative of treatment effect. Data for mean lesion volume, number of animals and standard deviation were extracted for treatment and control groups. All reported studies were included in analysis. Comprehensive meta-analysis software (version 2.2.064) was used for statistical analyses. Results were rechecked using MedCalc Version 12.0.3.0. Data was analyzed using two methods: (i) standardized mean difference method and (ii) ratio of means method.

In the first method, standard difference in mean represented by Hedge's g value is calculated. Standardized mean difference (SMD) is difference in the means of treatment (Mt) and control (Mc) group in multiples of pooled standard deviation (Sp). It is called as Cohen's d.

$$d = \frac{Mt - Mc}{Sp}$$

Thus, it is the difference in means in units of standard deviation.

Hedge's g is obtained by multiplying Cohen's d with a correction term which reduces bias caused by small sample size. Thus, $g = d(1 - 3/[4(Nt + Nc) - 9])$, where Nt and Nc are the sample sizes of treatment and control group, respectively. Meta-analysis was run using both fixed and random effect models. Weighted mean value for Hedge's g was calculated and 95% confidence interval was determined.

The results of analysis are shown in forest plot (Fig. 3a). The pooled effect size indicated that treatment with ET_A antagonist significantly reduced the infarct volume ($p \nabla 0.0001$). There exists considerable heterogeneity in the study results (Q value 29.41, degree of freedom 9, I^2 69.40). Therefore random effect model was applied for calculating pooled effect size. Publication bias was assessed by visually examining a funnel plot of standard error against the standard mean difference (Fig. 3b). Studies with poor precision (more standard error) were found to show more pronounced reduction in infarct volume, thus indicating a publication bias.

Ratio of means method is useful when treatment effects are expressed in different units. It also reduces bias that is observed in SMD method [91]. Briefly, ratio of mean infarct volumes of treatment and control groups were calculated. Their variance was estimated using delta method as described earlier [91]. Pooled mean ratio was calculated using random and fixed effect models. Infarct in the treatment group was found to be reduced to 60%. (95% CI 47–77%, $p \nabla 0.0001$). Alternatively percent reduction in lesion volume can be estimated by a slight modification of ratio of means method [92]. In this method, mean and standard deviation of treatment and control groups are divided by mean of control group. Thus, infarct volume obtained in control group is considered as unity or 100%. Percent reduction in infarct volume caused by treatment can be estimated by simply by calculating difference in the means. With this method, ET_A antagonist treatment was found to reduce the volume of infarction by 36.69% as compared with control (95% CI 24.26–49.12%, $p \nabla 0.0001$) (Fig. 4).

Overall, ET_A antagonists were found to show significant neuroprotective effect in focal cerebral ischemia. The neuroprotective effect was found to be affected by method of drug administration and the time of infarct volume assessment. It is important to note here that these studies showed the methodological rigor as per initial recommendations of STAIR committee only to some extent. In most of the studies, multiple dose regimens were evaluated. Physiological parameters were monitored during the surgical procedures. All studies reported evaluation of infarct volume and functional response. However, none of the studies reported the stroke model in aged animals or in animals with hypercholesteremia. Only exception was a study reporting efficacy of SB217242, in which spontaneously hypertensive rats were used. From the entire analysis, it was not possible to draw any conclusion regarding the time window for effective ET_A antagonist therapy. A recent update on STAIR recommendations emphasizes on sample size calculations, randomization and blinding of assessment [93]. Adherence to these recommendations was not reported in the literature that was analyzed.

A careful analyses of all these studies (Tables 2 and 3), suggests that ET_A antagonists have neuroprotective effects in focal as well as global cerebral ischemia. Dose and mode of drug administration, duration of ischemia, and time of assessment of infarct volume have an impact on the observed efficacy of ET_A antagonist. The neuroprotection is most likely to be evident in the form of reduction in infarct volume after 24 hours of ischemic episode. Neuroprotective effect of ET_A antagonist seems to evolve slowly. For this purpose it is necessary to maintain adequate concentrations of ET_A antagonist in the brain. The extent of neuroprotection observed after ET_A antagonist treatment should be viewed cautiously considering the quality of studies.

Possible mechanisms of neuroprotection

Reduction in post-ischemic hypoperfusion

Post-ischemic hypoperfusion mediated by contractile ET receptors is thought to worsen ischemic damage. ET_A antagonists are found to counteract delayed hypoperfusion. BQ123 treatment 30 min before 15 min after bilateral carotid artery occlusion (BCAO) abolished the post-ischemic hypoperfusion as well as CBF changes caused by NLA, competitive inhibitor of NO synthesis [84]. Similarly, BQ610 administered 15 min before BCAO improved microvascular perfusion in gerbils [85]. SB234551 and Ro 61-1790 treatment has also improved post-ischemic hypoperfusion in rats [53,54]. Significant improvement in CBF deficits along with reduction in infarct volume were also observed in MCA occluded cats treated with PD155707 [43].

Protection of penumbra

Improvement in perfusion is beneficial to penumbra region which is thought to be a salvageable tissue. The penumbra region can be identified through mismatches between tissue perfusion-weighted imaging (PWI; an index of blood flow deficit) and cellular

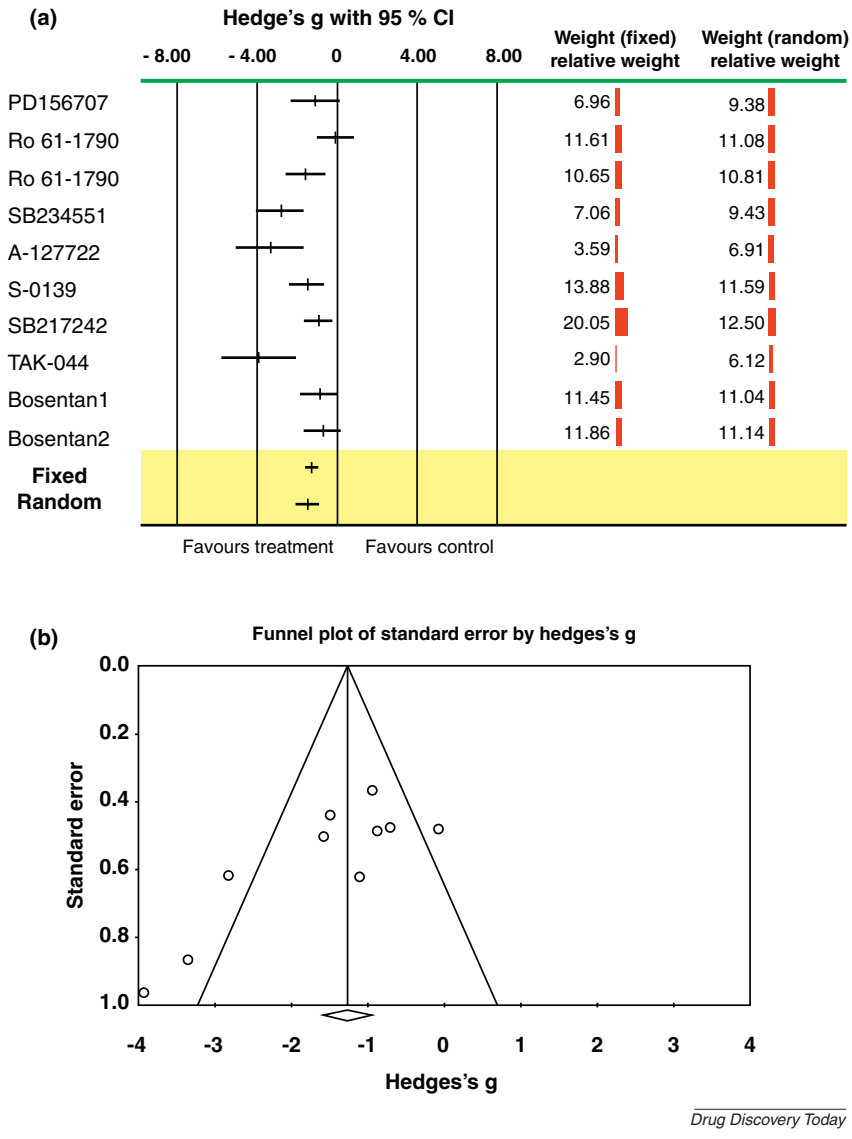

FIGURE 3

(a) Forrest plot showing Hedge's *g* (standardized difference in mean) with 95% confidence interval (CI). Effect size with fixed effect model $g = -1.26$ (CI −1.58, −0.94). Effect size with random effect model $g = -1.48$ (CI −2.08, −0.87). **(b)** Funnel plot of standard error against the Hedge's *g* showing the existence of publication bias.

diffusion-weighted imaging (DWI; an index of tissue injury). Neuroprotective effects of ET_A receptor antagonist is likely to be observed when it is administered during the time of PWI–DWI mismatch as salvageable tissue is present and the blood flow gets directed toward it. This is exemplified by SB234551, a selective ET_A antagonist, which shows its neuroprotective effect only in a stroke model with salvageable penumbra region. It fails to show neuroprotective effects when administered beyond the PWI–DWI mismatch period [94]. This suggests that the improved CBF might be one of the probable mechanisms in ET_A antagonists mediated neuroprotection in a model of stroke with salvageable penumbra. Perfusion and/or diffusion weighted MRI study further suggested that significant reduction in infarct volume observed in A127722 treated group could not be explained solely on the basis of improved perfusion and the possible mechanism could be inhibition of ET mediated excitotoxicity [44]. However, A127722 does not inhibit binding of MK-801 (NMDA receptor antagonist) to NMDA receptors. Thus, there is no possibility of direct glutamate receptor antagonism.

Reduction in BBB damage and anti-inflammatory effects

Post-ischemic ET release contributes to BBB disruption, edema formation and enhancement of infarction and these effects could be attenuated by ET_A antagonist treatment. ET antagonists were found to prevent albumin extravasation in the animal models of cerebral ischemia [34]. S-139 treatment not only attenuated BBB disruption but also showed a significant reduction in brain edema [34]. Anti-inflammatory potential of ET antagonists has also been explored in the cerebral ischemia. Lehmberg *et al.* demonstrated that leukocyte endothelium interactions were reduced by the pretreatment of BQ610 [85]. BSF-208075 has shown to reduce the post-ischemic leukocyte activation in ischemic brains [66].

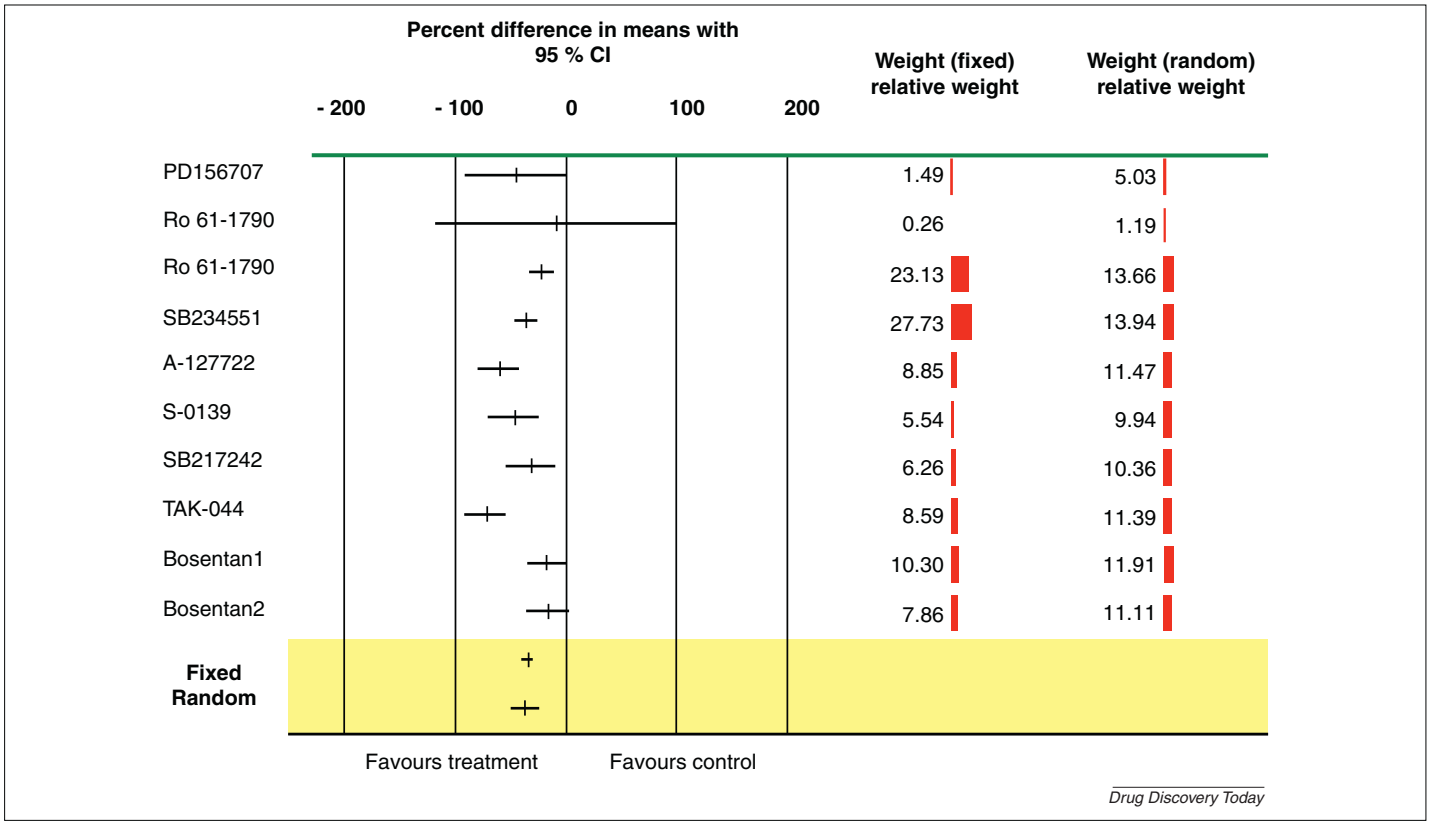

FIGURE 4

Forrest plot showing percent difference in means with 95% confidence interval (CI). Effect size with fixed effect model = −34.28 (CI −39.84, −28.72). Effect size with random effect model = −36.69 (CI −49.12, −24.26).

These examples suggest that ET antagonists might contribute to neuroprotection at least in part by ameliorating the inflammatory mechanisms in ischemic brains.

Combination therapy involving ET antagonists

Tissue plasminogen activator is the first line treatment for acute embolic stroke. Recombinant tPA (rtPA) is required to be used within 4.5 hours of embolic onset [95,96]. rtPA also causes hemorrhagic transformations, BBB damage and induction of ICAM and protease-activated receptor 1 (PAR-1) which are responsible for secondary thrombosis. Late therapy with rtPA also reduces the expression of tight junction protein namely collagen, laminin and occludin and ultimately disrupts the vascular integrity. ET receptor antagonists might yield synergistic effects when administered in combination with other neuroprotective agents. The combination therapy of S-0139 and rtPA significantly reduced infarct volume and hemorrhagic area and improved functional recovery. Combination therapy synergistically suppressed ischemia- and rtPA-induced ICAM-1, PAR-1 and loss of tight junction proteins in cerebral vessels [97]. However combination therapy of ET_A antagonist, FR139317 with a modified tissue-type plasminogen activator, SUN9216 failed to show benefits [98]. Type of model and duration of administration might be responsible for the observed incongruity between these two studies. S-0139 was evaluated in thrombo-embolic model whereas FR139317 was evaluated in photothrombotic model. Most importantly, S-0139 was administered as a continuous infusion lasting for 22 hours and this might have produced sustained effect of ET_A antagonism.

ZD1611, an ET antagonist and candesartan, AT-1 receptor antagonist did not show neuroprotection when administered as monotherapy in rat MCAO model. However, a combination of these two compounds decreased brain damage and improved neurological score. Interestingly, the combination did not alter the mean arterial blood pressure [99]. This could be of significance because it is desirable to improve cerebral perfusion without reducing peripheral blood pressure. So a rationally designed combination of ET antagonists with other potential neuroprotective agents could be a better therapeutic strategy.

Concluding remarks

Cerebrovascular origin of stroke propels the scientists to think about targets related with vasoregulation. Post-ischemic cerebral blood flow is the crucial factor in determining outcome of stroke. ET is the most potent vasoregulator known to man and has a role in regulating post-ischemic perfusion. Increased expression of ET peptide and its receptors is thought to exacerbate ischemic damage. Moreover, evidence suggests for a broader role of ET in post-ischemic pathogenic cascade. Therefore, many attempts have been made to target ET receptors in various animal models of stroke. These have shown promising results and suggested a useful neuroprotective strategy. However, these studies differ in many aspects, such as animal model, treatment schedule, parameters and techniques used for assessing these parameters. A meta-analysis of all studies in a model of focal cerebral ischemia indicates significant reduction in the lesion volume which subsequently results in improvement in functional outcome.

However, it is important to consider the obvious differences in animal models and clinical stroke. This poses a limitation on the conclusions we draw about the extent of neuroprotection observed with ET_A antagonists. Antagonism of ET_A receptor in post-ischemic brain is definitely of therapeutic benefit but extent of observed neuroprotection in animal models can be exaggerated. A rigorous analysis of the experimental models has shed a light on the exact role of ET antagonists as neuroprotectants. Conditions in which neuroprotective effect is observed and extent of this neuroprotection will together determine the utility of ET_A antagonists in the treatment of ischemic stroke. Improved hemodynamics along with attenuated cerebral edema after ET_A receptor antagonist treatment can be of vital benefits when combined with presently established recanalization therapy. ET_A receptor antagonists appear to offer an essential advantage of multiple neuroprotective mechanisms including prevention of BBB disruption and leukocyte infiltration. Taken together ET_A receptor antagonism stands as a promising neuroprotective strategy in ischemic stroke.

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