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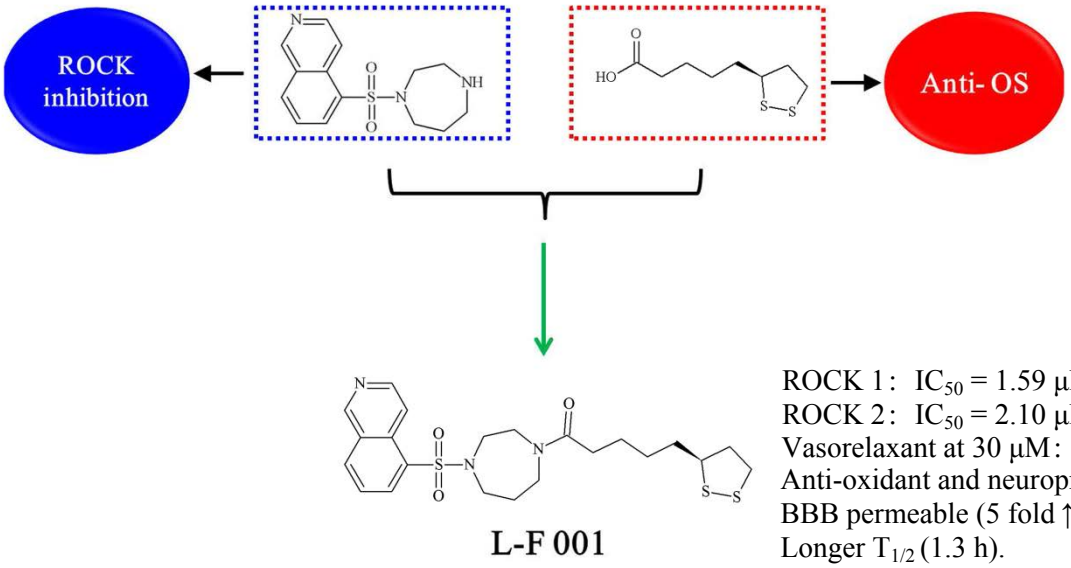
Graphical abstract



ROCK 1: IC_{50} = 1.59 μM,
ROCK 2: IC_{50} = 2.10 μM;
Vasorelaxant at 30 μM: 50%;
Anti-oxidant and neuroprotective agent;
BBB permeable (5 fold ↑);
Longer $T_{1/2}$ (1.3 h).

Journal Name

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COMMUNICATION

Simply combining Fasudil and Lipoic acid in a novel multitargeted chemical entity potentially useful in central nervous system disorders

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Current drugs against central nervous system (CNS) disorders have limited symptomatic activities, and new approaches with neuroprotective and neurorestorative properties are urgently needed. The complex pathology of CNS disorders requires the development of multitargeted or multifunctional drugs towards several CNS targets. In the present work, employing the pharmocophore of Fasudil, a Rho-associated coil kinase (ROCK) inhibitor, and alpha-Lipoic acid (LA), a potent anti-oxidant, we have developed a novel multitargeted and neuroprotective drug, L-F 001. L-F 001 displayed potent inhibition towards both ROCK 1 (IC_{50} = 1.59 μM) and ROCK 2 (IC_{50} = 2.10 μM) and reduced the actin stress formation. Rat thoracic aorta assay showed that L-F 001 exerted potent vasodilation. Furthermore, the compound was capable of scavenging free radicals, increasing the level of glutathione, and preventing HT 22 cell death caused by glutamate (Glu). Moreover, the new entity had higher brain permeation over Fasudil according to *in vitro* and *in vivo* blood-brain barrier (BBB) permeability tests. These results indicate that L-F 001 is a promising multifunctional agent for the treatment of CNS disorders.

Central nervous system (CNS) disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD), have been considered as one of the most problematic issues in biomedicine[1]. These diseases share some common pathways, including protein misfolding and oxidative stress[1]. The market value for PD and AD treatment exceeded $6.5 billion in 2009 and these CNS disorders will surpass cancer as the second cause of death in the elderly [2]. However, there is still an unmet need in such diseases. The present therapeutics for CNS disorders at best are symptomatic and are not able to delay disease or possess disease modifying activity. Therefore, new approaches with disease-modifying activity are urgently needed [2].

Rho-associated coil kinase (ROCK), one major downstream effector of the small GTPase, Rho, plays a key role in fundamental cellular functions such as cell motility, invasion, contraction, differentiation, migration and survival [3]. Abnormal activation of the Rho/ROCK pathway has been observed in several CNS disorders and ROCK inhibitors have shown to be beneficial for the treatment of such diseases [4]. Previous studies have pointed out that combining 5-aminoindazole or a 5-aminoisoquinoline-based ROCK inhibitor with a prostaglandin derivative into one molecule showed additive and/or synergistic effects in treating glaucoma [5]. We previously reviewed Fasudil, the only clinically available ROCK inhibitor showed beneficial effects in animal models and/or clinical applications for CNS disorders, and by conjugating another pharmacophore with Fasudil could generate new entity with multifunctional and mild adverse effects, thus provide better clinical application[6].

Increased Oxidative stress has been considered as a common culprit of many CNS disorders[7]. α-Lipoic acid (LA), readily obtained from the diet, has been termed a "universal antioxidant". It is capable of scavenging a number of free radicals and is found to cross blood-brain barrier (BBB)[8]. Thus, LA could be considered a privileged structure in designing new multitarget-directed ligands (MTDLs) for the investigation and, conceivably, treatment of CNS disorders[9].

Drugs hitting one single target may be inadequate for the treatment of CNS disorders involving multifaceted factors and a possible paradigm shift in drug design is to create molecules that can hit multiple targets such as MTDLs[2]. Recent studies have pointed out that the strategy of targeting two or more targets with one compound can provide therapeutic effects superior to those of a selective drug [2].

Scheme 1. Synthesis of L-F 001.

Given the above mentioned substantial body of evidence, it could be argued that blocking ROCK and scavenging ROS could constitute a high-priority in treating CNS disorders. Inspired from this rationale, we incorporated the anti-oxidative LA moiety into the ROCK inhibitor Fasudil moiety to develop a multitargeted compound L-F 001 (Scheme.1.). The present study reports that a

novel multitargeted chemical entity, L-F 001 by simply combining Fasudil and LA, may be a promising multifunctional agent for the treatment of CNS disorders.

To investigate the *in vitro* ROCK inhibition of L-F 001, we applied Z'-LYTE kinase assay, a biochemistry method [10]. Fasudil showed potent inhibition against ROCK 1 (IC_{50} 0.66 μM) and ROCK 2 (IC_{50} 0.21 μM), similar as previously reported [4], while L-F 001 displayed milder activity but still potent enough to block ROCK 1 (IC_{50} 1.59 μM) and ROCK 2 (IC_{50} 2.10 μM) (Table 1). To provide insights into the enzyme–inhibitor interaction, we performed molecular docking experiment. Fig. S1 showed the binding mode of L-F 001 with ROCK 1. The result was consistent with kinase inhibitory assay. It seemed that the introduction of LA moiety into Fasudil resulted in slightly decrease of the selectivity.

Table 1 IC_{50} (μM) of Fasudil and L-F 001 against ROCK 1/2.

Compound	ROCK 1	ROCK 2
Fasudil	0.66	0.21
L-F 001	1.59	2.10

ROCK plays a key role in remodeling the actin cytoskeleton under certain stimulus including high glucose, and inhibition of ROCK reversed such actin stress formation[11]. We performed rhodamine-phalloidin staining by laser scanning confocal microscopy (LSCM) to investigate the formation of actin stress fiber. Our control experiments showed that high glucose in deed enhanced actin stress fiber formation (Fig.1.). And there was an marked reduction of high glucose-induced actin stress formation in cells pre-treated with Fasudil and L-F 001 in HT 22 cells.

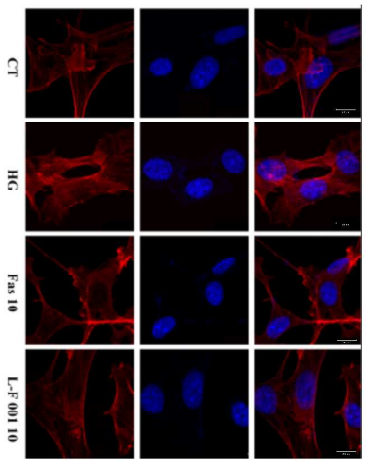

Fig. 1. L-F 001 decreased actin stress formation caused by high glucose medium. HT 22 cells were pretreated with L-F 001 (30 μM) and Fasudil (30 μM) for 0.5 h and subsequently stimulated with high glucose (30 mM). Red fluorescence indicates localization of F-actin. Blue fluorescence indicates nuclei. Scale bar represents 20 μM.

To our knowledge, Fasudil is a clinically used vasodilative drug with significant vasorelaxant effects [12]. Therefore we elucidated the vasorelaxant effects of L-F 001 by a rat aorta assay. Contractile response of aorta was induced by adding KCl (80 mM) into the organ bath. As shown in Fig. S2., compared with the DMSO control group, Fasudil induced a 70% relaxation at 30 μM and L-F 001 had a milder relaxation (45%) at the same concentration, while no vasodilating effects was exerted by LA. Considering the fact that the strong vasodilation of Fasudil usually leads to side effects, such as dilating peripheral vessel in clinically treatment of subarachnoid hemorrhage, it is possible that L-F 001, with a milder vasodilation compared with Fasudil, might avoid the side effects caused by Fasudil at some extent.

LA exhibits its antioxidant activity not only by directly scavenging radical, but also by regenerating other antioxidants, including glutathione (GSH)[13]. Furthermore, LA is able to cross BBB and to enhance the availability of a compound conjugated with LA in the brain[14]. Therefore, we elucidated the effects of L-F 001 on ROS production and endogenous antioxidants using fluorescent probe H_2-DCF-DA and a GSH assay kit. Flow cytometric analysis revealed that Glu caused a marked increase of ROS, and L-F 001 and LA profoundly scavenged ROS at 30 μM (Fig. 2A.), while Fasudil had limited effects. Next, we measured the effects of L-F 001 on intracellular GSH level. Results showed that Glu greatly decreased the intracellular level of GSH as previously reported[15], while L-F 001 and LA, but not Fasudil, significantly reversed the process.

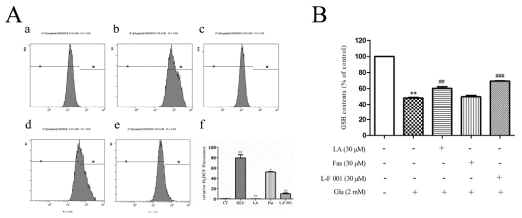

Fig. 2. L-F 001 exerted potent anti-oxidative effects by scavenging ROS and increasing intracellular GSH level. HT 22 cells were treated with LA, Fasudil, and L-F 001 at 30 μM for 0.5 h, and further exposed to Glu (2mM) for 10 h. (A) The ROS level of HT 22 cells were evaluated by DCF fluosence. a) CT; b) Glu; c) LA+ Glu; d) FAS+ Glu; e) L-F 001+ Glu; f) Quantitative analysis for relative DCF flurosence. (B) The level of GSH was measured using commercial assay kits. The basal contents of GSH in untreated control cells were taken as 100%. $^{**}P<0.01$, compared with the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the cells treated with Glu alone (n = 6).

Glutamate (Glu) is reported to be neurotoxicity, and neuroprotective drugs are able to protect cells against its toxicity[16]. Therefore, we examined the cytoprotective effects of L-F 001 against Glu-induced toxicity by MTT assay. Results indicated L-F 001 and LA, but not Fasudil, dose-dependently decreased Glu-induced cytotoxicity (Fig. S3B.). Results of cell morphology, photographed under phase-contrast optics were consistent with MTT assay (Fig. S3A.).

Brain penetration is a key factor for successful drugs for treatment of CNS disorders[17]. The physico-chemical properties of drugs control the penetration through the biological membranes, and their evaluation may be useful in order to understand the pharmacokinetics profile of drugs employed as neuroprotective agents. The lipophilicities of the compounds were approximated by clogP values, calculated by ACD Log P/ Log D prediction

software (version 4.55, Advanced Chemistry Development Inc., Toronto, Canada). L-F 001 (cLog P 3.4 ± 0.9) showed higher lipophilicities than Fasudil (cLog P 1.2 ± 0.8) as shown in table 2. To evaluate the potential for these hybrids to cross the BBB, we used a parallel artificial membrane permeation assay for BBB (PAMPA-BBB), which was described by Yang Sun *et al* [18]. Assay validation was carried out by comparing experimental permeabilities of 13 commercial drugs with reported values. The results showed that L-F 001 exhibited good permeability through PAMPA membranes and could be classified as potently brain permeable agents ($P_e \geq 4.7 \times 10^{-6}$ cm s^{-1}), while Fasudil showed low BBB permeability ($P_e < 2.0 \times 10^{-6}$ cm s^{-1}) which was considered low BBB permeation. Next, in order to evaluate the *in vivo* BBB permeation, the concentrations of Fasudil and L-F 001 in the plasma and brain homogenate were examined after administration of the two drugs. The conjugate exhibited 5-fold higher brain concentration when compared with an equimolar dose of Fasudil alone. The results suggested that L-F 001 might target mainly in CNS and possess less peripheral effects, thus providing better clinical application than Fasudil in the treatment of CNS disorders.

Table 2 L-F 001 exhibited better BBB permeation than Fasudil.

compound	cLogP	Permeability ($P_e \times 10^{-6}$ cm s^{-1})	Prediction*	$R_{Brain/Blood}$#
Fasudil	1.2 ± 0.8	1.0 ± 0.2	CNS -	0.1 ± 0.2
L-F 001	3.4 ± 0.9	6.3 ± 0.5	CNS +	0.5 ± 0.1

*CNS^+: ($P_e \times 10^{-6}$ cm s^{-1}) > 4.7, CNS^-: ($P_e \times 10^{-6}$ cm s^{-1}) < 2.0[18].
see supporting information **13**.

The pharmacokinetic parametersindicated that L-F 001 was rapidly absorbed in rats (Table S1).Time of peak concentration is 42 min. Following administration of L-F 001 to male rats, a relatively high exposure (C_{max} 651 nM, $AUC_{0\rightarrow t}$: 793.6 ng·h/mL, $AUC_{0\rightarrow\infty}$: 805.5 ng·h/mL), a rapid absorption (T_{max}: 0.7 h) were observed. Interestingly, L-F 001 had a longer $T_{1/2}$ (1.3 h) than LA (0.4 h)[19] and Fasudil (0.5 h)[20].

In summary we have developed a multitargeted drug L-F 001 obtained by chemically combining Fasudil and LA, two compounds hitting different targets for CNS disorders. Our new chemical entity turned out to be remarkably active against ROCK, reduce actin stress formation and dilate contracted vessels, which were comparable with Fasudil. In addition, L-F 001 also could scavenge ROS, reverse GSH depletion and protect neuronal cells from Glu toxicity similar with LA. Moreover, the new entity was able to cross BBB as provided by clog P data and PAMPA-BBB, and confirmed by *in vivo* BBB permeation test.

By one simple step, we obtained L-F 001, a novel chemical entity, which was a potent ROCK inhibitor with anti-oxidative and neuroprotective effects. Our study showed that it was able to cross brain-blood barrier and possess multifunctional effects, suggesting that L-F 001 might be a promising drug candidate for CNS disorders, where both free radical damage and abnormal activation of the Rho/ROCK pathway are involved.

We acknowledge the Guangdong Provincial International Cooperation Project of Science& Technology (No. 2012B050300015), National Natural Science Foundation of China (No.31371070), and Scientific and Technological Cooperation between the Italian Republic and the People's Republic of China for Year 2013-2015 (No. MAE-M00705\CN13MO9)

Notes and references

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1. A. Cavalli, M. L. Bolognesi, A. Minarini, M. Rosini, V. Tumiatti, M. Recanatini and C. Melchiorre, *Journal of medicinal chemistry*, 2008, **51**, 347-372.
2. M. B. Youdim, *Experimental neurobiology*, 2010, **19**, 1-14.
3. F. Amigoni, E. Legnaghi and P. Pevarello, *Pharmaceutical patent analyst*, 2012, **1**, 177-192.
4. P. Pan, M. Shen, H. Yu, Y. Li, D. Li and T. Hou, *Drug discovery today*, 2013, **18**, 1323-1333.
5. Y. Feng and P. V. LoGrasso, *Expert opinion on therapeutic patents*, 2014, **24**, 295-307.
6. M. Chen, A. Liu, Y. Ouyang, Y. Huang, X. Chao and R. Pi, *Expert opinion on investigational drugs*, 2013, **22**, 537-550.
7. M. Valko, D. Leibfritz, J. Moncol, M. T. Cronin, M. Mazur and J. Telser, *The international journal of biochemistry & cell biology*, 2007, **39**, 44-84.
8. J. J. Harnett, M. Auguet, I. Viossat, C. Dolo, D. Bigg and P. E. Chabrier, *Bioorganic & medicinal chemistry letters*, 2002, **12**, 1439-1442.
9. M. Rosini, E. Simoni, M. Bartolini, A. Tarozzi, R. Matera, A. Milelli, P. Hrelia, V. Andrisano, M. L. Bolognesi and C. Melchiorre, *European journal of medicinal chemistry*, 2011, **46**, 5435-5442.
10. R. Pireddu, K. D. Forinash, N. N. Sun, M. P. Martin, S. S. Sung, B. Alexander, J. Y. Zhu, W. C. Guida, E. Schonbrunn, S. M. Sebti and N. J. Lawrence, *MedChemComm*, 2012, **3**, 699-709.
11. X. Xie, J. Peng, X. Chang, K. Huang, J. Huang, S. Wang, X. Shen, P. Liu and H. Huang, *Molecular and cellular endocrinology*, 2013, **369**, 86-97.
12. G. J. Velat, M. M. Kimball, J. D. Mocco and B. L. Hoh, *World neurosurgery*, 2011, **76**, 446-454.
13. M. Jalali-Nadoushan and M. Roghani, *Brain research*, 2013, **1505**, 68-74.
14. S. Zara, M. De Colli, M. Rapino, S. Pacella, C. Nasuti, P. Sozio, A. Di Stefano and A. Cataldi, *Gerontology*, 2013, **59**, 250-260.
15. D. Zhu, M. Chen, M. Li, B. Luo, Y. Zhao, P. Huang, F. Xue, S. Rapposelli, R. Pi and S. Wen, *European journal of medicinal chemistry*, 2013, **68**, 81-88.
16. E. Poteet, A. Winters, L. J. Yan, K. Shufelt, K. N. Green, J. W. Simpkins, Y. Wen and S. H. Yang, *PloS one*, 2012, **7**, e48279.
17. D. E. Clark and S. D. Pickett, *Drug discovery today*, 2000, **5**, 49-58.
18. Y. Sun, J. Chen, X. Chen, L. Huang and X. Li, *Bioorganic & medicinal chemistry*, 2013, **21**, 7406-7417.
19. J. Chen, W. Jiang, J. Cai, W. Tao, X. Gao and X. Jiang, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, 2005, **824**, 249-257.
20. H. Chen, Y. Lin, M. Han, S. Bai and S. Wen, *Journal of pharmaceutical and biomedical analysis*, 2010, **52**, 242-248.