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A novel serine racemase inhibitor suppresses neuronal over-activation *in vivo*

Hisashi Mori [a,d,*], Ryogo Wada [b], Satoyuki Takahara [c], Yoshikazu Horino [c], Hironori Izumi [d], Tetsuya Ishimoto [d], Tomoyuki Yoshida [a,d], Mineyuki Mizuguchi [a,d], Takayuki Obita [d], Hiroaki Gouda [e,*], Shuichi Hirono [f], Naoki Toyooka [b,c,*]

[a] *Graduate School of Innovative Life Science, University of Toyama, Toyama 930-0194, Japan*
[b] *Graduate School of Science and Engineering, University of Toyama, Toyama 930-8555, Japan*
[c] *Graduate School of Innovative Life Science, University of Toyama, Toyama 930-8555, Japan*
[d] *Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan*
[e] *School of Pharmacy, Showa University, Tokyo 142-8555, Japan*
[f] *School of Pharmacy, Kitasato University, Tokyo 108-8641, Japan*

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ABSTRACT

Serine racemase (SRR) is an enzyme that produces D-serine from L-serine. D-Serine acts as an endogenous coagonist of NMDA-type glutamate receptors (NMDARs), which regulate many physiological functions. Over-activation of NMDARs induces excitotoxicity, which is observed in many neurodegenerative disorders and epilepsy states. In our previous works on the generation of SRR gene knockout (Srr-KO) mice and its protective effects against NMDA- and Aβ peptide-induced neurodegeneration, we hypothesized that the regulation of NMDARs' over-activation by inhibition of SRR activity is one such therapeutic strategy to combat these disease states. In the previous study, we performed *in silico* screening to identify four compounds with inhibitory activities against recombinant SRR. Here, we synthesized 21 derivatives of candidate **1**, one of four hit compounds, and performed screening by *in vitro* evaluations. The derivative **13J** showed a significantly lower IC_{50} value *in vitro*, and suppressed neuronal over-activation *in vivo*.

1. Introduction

In mammals, a relatively high amount of free d-serine, which is not used for protein synthesis, is detected mainly in the forebrain.[1] d-Serine is produced from l-serine by serine racemase (SRR), a pyridoxal phosphate (PLP)-dependent enzyme.[2] d-Serine acts as an endogenous coagonist of NMDA-type glutamate receptors (NMDARs). Full activation of NMDARs requires simultaneous binding of the agonist glutamate and coagonist d-serine or glycine. NMDARs are involved in many physiological and pathological states including neural network formation, synaptic plasticity, learning and memory, neurodegenerative disorders, and psychiatric disorders.[3] Over-activation of NMDARs induces excitotoxicity, which is observed in many neurodegenerative disorders and epilepsy states. We have generated SRR gene knockout (Srr-KO) mice and revealed the protective phenotypes of the Srr-KO mice against NMDA- and Aβ peptide-induced neurodegeneration.[4] Thus, the regulation of NMDARs' over-activation by the inhibition of SRR activity is one such therapeutic strategy to combat these disease states. Malonate was reported as the most popular SSR inhibitor,[5] followed by several other inhibitors such as dipeptides,[6] hydroxamic acid derivatives,[7] and malonate-based derivatives.[8] However, the *in vivo* effect of these inhibitors has not been reported.

Previously, we identified SRR inhibitor candidates using *in silico* screening based on the reported three-dimensional structure of SRR.[9] Table 1 shows the inhibitory activities of these compounds using recombinant mouse SRR. In our previous study, we also reported novel chemicals possessing SRR inhibitory activities comparable to malonate, based on the candidate **4** (Fig. 1).[10]

In this study, we attempted to obtain novel compounds with more potent SRR inhibitory activities than malonate, based upon the remaining candidates. We specifically focused on derivatives of compound **1**, as its inhibitory activity was the most potent among the four compounds. Next, to extend our study *in vivo*, we employed the *Arc-Luc* transgenic (Tg) mouse strain.[11] Using the *Arc-Luc* Tg hairless (HL) mouse strain, we can examine the change in neuronal-activity-dependent expression of the *Arc* gene with

* Corresponding authors at: Graduate School of Innovative Life Science, University of Toyama, Toyama 930-0194, Japan (H. Mori). Graduate School of Science and Engineering, University of Toyama, Toyama 930-8555, Japan (N. Toyooka).
E-mail address: toyooka@eng.u-toyama.ac.jp (N. Toyooka).

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Table 1
Results of *in vitro* assay of **1**–**4** using mouse SRR. The remaining activity of SRR with the inhibitors (1 mM) was evaluated with the percentage of the D-serine production compared with that without inhibitors.

Compound	Activity [%]
1	27
2	32
3	36
4	35

bioluminescent signals. The expression of *Arc* is dependent on the activity of NMDARs,[12] thus, we can monitor the activity of NMDAR in the *Arc-Luc* Tg HL mice with the change in the bioluminescent signal from a live mouse. Using this technique, we successfully identified a novel SRR inhibitor suppressing the *Arc* expression *in vivo*.

2. Results and discussion

2.1. Modeling of interaction mode of *1* with SRR

First, in order to obtain a strategy for designing derivatives of **1**, the interaction mode of **1** with SRR was investigated by the previously constructed procedure,[10] which consisted of the molecular-docking calculation and molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) free energy analysis.[13] The resulting binding mode of **1** with SRR is shown in Fig. 2.

SRR exists in a dimeric form. Therefore, we denote one chain as "A" and the other chain as "B." The central part of **1**, the acyl hydrazino thiourea moiety, is suggested to be significantly important to interact with SRR, as it formed a total of four hydrogen bonds with N86, D238, and N316 of the A chain of SRR (Fig. 2**A**). The thiophene moiety of **1** was located in the malonate binding site of the A chain of SRR to create van der Waals interactions with PLP-K56, S83, S84, G85, N86, and G239. On the other hand, the phenyl moiety of **1** oriented in the opposite direction and created van der Waals interactions with R277, E276, and K279 of the B chain of SRR. This means the phenyl moiety of **1** is located in the interface region of the dimer. As shown in Fig. 2**B**, available spaces are visible around the thiophene and phenyl moieties of **1**. Therefore, we replaced these two moieties with larger substituents to design more potent derivatives of **1**, aiming at the increase of the van der Waals interaction.

2.2. Chemistry

According to the above information obtained by the interaction mode of **1** with SRR, we designed the derivatives of the most potent candidate **1** based on the central structure of acyl hydrazino thiourea moiety **1D** (Fig. 3) of **1**.

Compounds **13A**–**U** were synthesized as shown in Scheme 1. The aldehydes **5a**–**h** were converted to unsaturated carboxylic acids **7a**–**h** by the Doebner condensation or the Horner-Wadsworth-Emmons reaction of the corresponding aldehydes **5a**–**h** followed by hydrolysis of the resulting esters **6g**, **h**. Conversion of **7a**–**h** to the corresponding acid chlorides, which were treated with NH_4SCN in the presence of catalytic amounts of PEG-400[14], afforded the isothiocyanates **8a**–**h**. The isothiocyanates **10c, i** were also prepared from the carboxylic acids **9c, i** in the same manner as that for **8a**–**h**. However, the hydrazides **12j**–**r** were prepared from the carboxylic acids **11j**–**r** via the corresponding esters with hydrazine. Finally, the addition reaction of the hydrazides **12j**–**r** to isothiocyanates **8a**–**h** and **10c, i** gave rise to the desired thioureas **13A**–**U**.

We also synthesized 35 and 37 derivatives of the candidates **2** and **3**, respectively. Like the chemical structure of **1**, both **2** and **3** possess several hydrogen bonding and two aromatic groups in their central and edge parts, respectively. Therefore, we adopted a similar strategy to that for **1** for designing derivatives of **2** and **3**. With the *in vitro* screening of these derivatives, we identified the derivatives **14** and **15** as the possible inhibitors of SSR. (Scheme 2) The derivative **14** possesses an amide linkage instead of ester functionality in **2**, and the derivative **15** possesses phenylenediamine structure instead of ethylenediamine unit in **3**. As shown in Fig. 4, the derivatives **14** and **15** showed almost same IC_{50} value (0.27 mM and 0.28 mM, respectively).

2.3. Evaluation of SRR inhibitory activities of derivatives of *13A–U, 14, and 15*

The synthesized derivatives **13A–U, 14, and 15** were assayed *in vitro* with recombinant human SRR with C2D2 mutations for which the X-ray crystal structure was reported[9] and that we used previously (Table 2).[10]

According to the *in vitro* evaluations of all synthesized derivatives, we selected three potent derivatives **13J**, **14**, and **15**. Although compounds **13A**-**B** are also highly potent inhibitors, the structures of these compounds are very similar to that of the original compound **1**. From the structural diversity, we expected to be able to identify a novel inhibitor. For these reasons, we examined the inhibitory effects of **13J**, **14**, and **15** in more detail, and determined the IC_{50} values of these compounds. Among three compounds, two of them (**14**, **15**) showed similar IC_{50} values to that of malonate, one of the standard inhibitors of SRR (Fig. 4). Another compound named **13J** showed a significantly lower IC_{50} value than that of malonate (Fig. 4).

13J possesses the phenyl and 3,5-dibromophenyl moieties, which are larger than the thiophene and phenyl moieties of **1**, respectively.

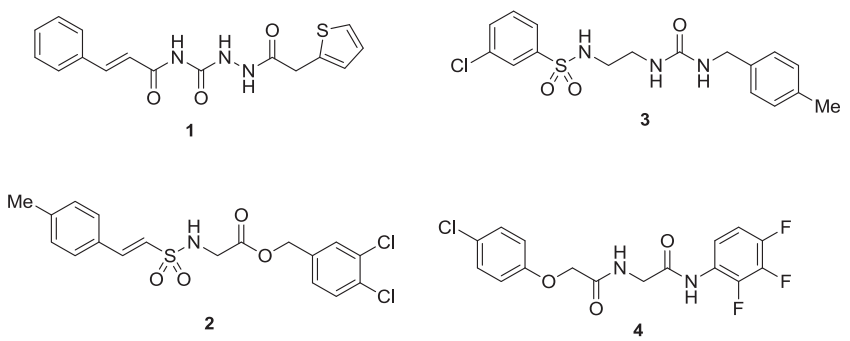

Fig. 1. Structure of four small molecules with inhibitory activity against SRR from eighteen virtual hits.