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# Riluzole-triazole hybrids as novel chemical probes for neuroprotection in Amyotrophic Lateral Sclerosis.

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**KEYWORDS** Amyotrophic lateral sclerosis, motor neuron disease, riluzole, primary cortical neurons.

**ABSTRACT.** Despite intense attention from biomedical and chemical researchers, there are few approved treatments for amyotrophic lateral sclerosis (ALS), with only riluzole (Rilutek<sup>®</sup>) and edaravone (Radicava<sup>®</sup>) currently available to patients. Moreover, the mechanistic basis of the activity of these drugs is currently not well-defined, limiting the ability to design new medicines for ALS. This manuscript describes the synthesis of triazole-containing riluzole analogues, and their testing in a novel neuroprotective assay. Seven compounds were identified as having neuroprotective activity, with two compounds having similar activity to riluzole.

## Introduction

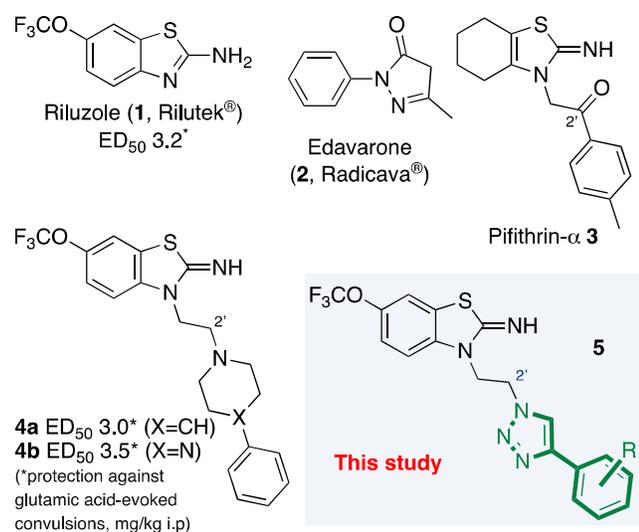
Amyotrophic lateral sclerosis<sup>1</sup> ('ALS', also known as motor neuron disease, or Lou Gehrig's Disease) involves a progressive loss of motor neurons in the central nervous system that controlling motor functions, usually resulting in death within 3-5 years after diagnosis. Though a rare condition (lifetime incidence ca. 1 in 400<sup>2</sup>), the disease places enormous social<sup>3</sup> and financial burdens on both patients<sup>4</sup> and healthcare programs. The condition is incurable, with riluzole<sup>5</sup> (**1**, Rilutek<sup>®</sup>) and edaravone<sup>6</sup> (**2**, Radicava<sup>®</sup>) the only drugs currently approved for treatment: both of these drugs provide short-term palliation, typically extending life by 2-3 months. In the case of familial ALS (accounting for 5-10% of cases) there is a genetic mutation which is causative,<sup>7</sup> but there is currently no consensus on the mode of action of the approved drugs, nor agreement on the key cellular targets for new drug-like neuroprotective molecules; thus, there is both scientific and practical value in the design and production of novel chemical matter delivering either mechanistic information or therapeutic activity for ALS.<sup>6, 8</sup> In addition to the two approved drug substances, there is currently a range of small molecule drug candidates<sup>9</sup> (including peptides,<sup>10</sup> saturated<sup>11</sup> and aromatic<sup>12</sup> heterocycles) being

studied in trials; however, there is still a relative paucity of entirely novel chemical matter for application in ALS therapy.

Riluzole-like heterocyclic small molecule frameworks are also known to demonstrate potential neuroprotective properties; thus, pifithrin- $\alpha$  (**3**),<sup>13</sup> which contains a partially saturated bicyclic thiazolyl core (rather than the benzothiazolyl scaffold seen in riluzole), and an aromatic substituent placed at the end of a C<sub>2</sub>-tether attached at the N<sub>1</sub> position. A similar substituent pattern is seen in riluzole analogues **4a** and **4b** (Figure 1)<sup>5</sup> with the presence of a functionalized ethyl side-chain endowing neuroprotection activity at similar levels to riluzole in rodent models.<sup>5</sup> As a novel chemical target with potential for neuroprotection, and bearing in mind the pyrazinone core of edaravone, we have synthesised previously unreported triazoles **5** as new chemical matter with structural resemblance to riluzole and related molecules.

The synthetic strategy to these compounds revolved around previously unknown lynchpin azide **6**, which would undergo copper(I)-catalysed cycloaddition with a range of alkynes to give **5** (Scheme 1). In addition to the triazole functionality being a known pharmacophoric motif in marketed drug substances (such as tazobactam and

cefatrizine), we anticipated that the modularity of the Click process also would allow for incorporation of other functionality (diazirine, dye labels etc.), thereby facilitating future mechanistic studies.

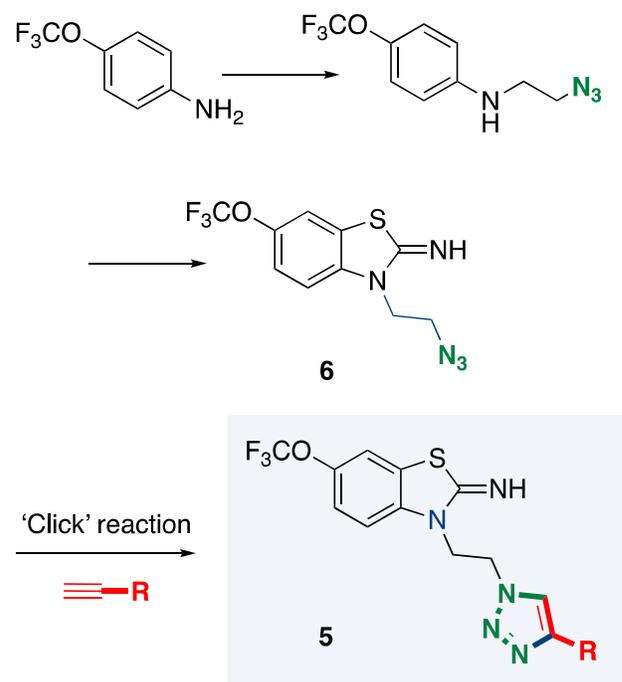


**Figure 1.** ALS drugs (**1** and **2**), neuroprotective small molecules (**1** and **2**), and project target (**5**)

A major challenge in the design of new therapies for ALS is the fact that the binding site of riluzole remains elusive, precluding definitive conclusions to be reached about the mode of action of approved drug substances and novel chemical matter alike. Previous studies using radioligand binding failed to demonstrate any interactions between riluzole and known ligand binding sites on the kainate and NMDA receptors, or the GABA<sub>A</sub> ( $\gamma$ -aminobutyric acid) or glycine receptors.<sup>14, 15, 16</sup> *in silico* docking analysis highlighted the Na<sub>v</sub>1.6 channel<sup>17</sup>. Since conclusive data to confirm the mechanism of action and binding modes of riluzole remain elusive, limiting the further development of riluzole in the therapeutic pipeline, we envisage tagged chemical probes will facilitate subsequent mechanistic studies once activity has been established.

A complication of the methods used to study the design and development of chemotherapeutics is the fact that the experimental preparations used to determine the mechanism of action of existing and new drugs and drug-like substances vary considerably, with many species, cell types protocol in use. Significantly, many of the cell lines used in the study of ALS are different to the cell

types primarily affected in the disease. This presents a challenge in direct comparison of data, and makes it difficult both to identify new drugs and to delineate mechanistic rationales. We describe here the synthesis of a novel library of hybrid small molecules containing features of both riluzole and edaravone, and the screening of this new chemical matter in novel neuroprotective assays.



**Scheme 1.** Click chemical strategy for preparation of triazole library **5**.

### Experimental details

In order to screen riluzole derivatives, and because of the paucity of robust *in vitro* assays in this field it was important to first determine the most suitable cell type, stimulus of cell death and measure of cell death to measure neuroprotection. Mouse primary cortical neurons and primary motor neurons were cultured using previously described methods<sup>18</sup>. For screens, neuroprotection assays based on MTT turnover, cell counting, caspase activation were discarded as riluzole or derivatives were not protective in these assays of cell death (results not shown). Since dendritic damage and loss is a cardinal feature of the slow degeneration found in motor neuron disease states we developed morphometric analyses to measure neuronal complexity following disease-

1 relevant stimuli. From a number of challenges  
2 tested, the AMPA receptor agonist, kainate was  
3 selected to elicit cell damage without rapid cell  
4 death. For primary cortical neurons an antibody  
5 against microtubule-associated protein, MAP2  
6 was used in Western blotting experiments and  
7 also to stain neuronal dendritic processes. Volocity  
8 software was used for perimeter analysis of  
9 cell profiles stained with MAP2 and each exper-  
10 imental condition was normalized to the kainate  
11 only condition.

12  
13 *Chemical matter.* The target triazole library was  
14 accessed in short order on hundred-milligram  
15 scale according to the synthetic strategy shown in  
16 Scheme 1. Thus, Click reaction of the previously  
17 unreported lynchpin azide **6** with a range of al-  
18 kynes, directly delivered previously unreported  
19 triazoles **5a-5aab** (Table 1). Armed with this li-  
20 brary of novel chemical matter, we proceeded to  
21 the testing program using *in vitro* model systems.

## 22 Results

23  
24 The library of riluzole derivatives **5a-5aab** were  
25 tested on primary cortical neurons for their ability  
26 to protect against kainate induced dendritic loss.  
27 From this assay, seven compounds were identi-  
28 fied as having promise (Figure 2, supporting in-  
29 formation). Test compounds **5ap**, **5ao**, **5r**, **5g**,  
30 **5aq**, **5w** and **5ak**, but not the parent, riluzole (ril),  
31 attenuated kainate-induced neurofilament loss.  
32 The remaining members of the triazole library  
33 did not prevent kainate-induced neurofilament  
34 loss (data not shown).

35  
36 The seven compounds that were positive in the  
37 MAP2 assay were also screened in primary corti-  
38 cal neurons, using plate-based technology to as-  
39 say functional properties. In healthy neurons  $K^+$   
40 can be used to depolarise neurons and initiate a  
41  $Ca^{2+}$  flux through voltage gated calcium chan-  
42 nels. This flux was monitored through incubating  
43 cells with fura-2AM for 45 mins, then washing  
44 and challenging with 10 mM KCl to induce cal-  
45 cium entry. Neurons pretreated with kainate ex-  
46 hibited a reduced calcium flux, indicating com-  
47 promised cell function. Riluzole and all the rilu-  
48 zole derivatives were able to attenuate this effect,  
49 with **5ap** and **5g** having the greatest effect (re-  
50 sults not shown).

51 We next determined protection using a secondary  
52 screen of primary mouse spinal cord motor neu-

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rons using the positive compounds from the cor-  
tical neuron screen. We first tested a range of  
outcome assays including cell counting and plate  
reading of SMI-32 fluorescence, but none pro-  
vided an output that was both sensitive to dis-  
ease-relevant stimuli and rescued by riluzole. We  
therefore employed the antibody SMI-32<sup>19</sup> to la-  
bel neurofilaments of motor neurons, and Sholl  
analysis<sup>20</sup> to monitor neuronal complexity. Fol-  
lowing treatments, cells were fixed and stained  
with SMI-32 antibody and fluorescent images of  
cells were captured. Ten individual cells were  
analysed for each condition, and the experiment  
was repeated three times and the data pooled. For  
Sholl analysis, concentric circles at each 100  $\mu$ m  
radius from the cell centre were drawn, and the  
number of intersections of processes at each radi-  
us interval were measured using image analysis  
software. The data provided an indication of the  
arborization and complexity of the motor neuron  
processes, with reduced process complexity and  
length preceding cell death. Figure 3 (supporting  
information) shows representative images and  
analysis from these experiments. Riluzole  
showed a significant neuroprotective effect, as  
did two of the test compounds, **5ap** and **5ao**.

## Discussion

*Phenyl derivatives.* Phenyl triazole **5a** was capa-  
ble of preventing kainate-induced MAP2 fluores-  
cence loss above that of the parent compound,  
riluzole. However triazoles in which the aryl mo-  
tif was placed at increasing distances from the  
heterocycle unit: increasing the length of the car-  
bon chain between the triazole and phenyl ring  
were not effective, with compound **5b** (placed at  
the terminus of a 2-carbon tether) and **5at** (with a  
3-carbon tether) showing reduced neuroprotec-  
tion compared with parent **5a**. Compound **5a** was  
not effective in the secondary screen in motor  
neurons.

*Substituted phenyl derivatives.* In compounds **5g-5l**  
the spatial arrangement of the carbon tether is  
changed, with the saturated motif placed on the  
phenyl ring as opposed to the triazolyl unit.  
Compound **5g**, bearing a *para*-C<sub>2</sub>-substituent  
showed significant activity against kainate-  
induced loss in MAP2 in cortical neurons while  
compounds **5h**, **5i**, **5k**, and **5l** (bearing linear *pa*-  
*ra*-carbon chains of 6, 5, 4, and 3 carbons, re-

spectively) did not significantly increase MAP2 fluorescence above that of kainate alone. Compound **5g** attenuated kainate reduction of calcium flux, but was not effective in the secondary screen in motor neurons.

*Alkyl triazoles.* Deletion of the phenyl ring of the scaffold and replacement with alkyl substituents (**5aw**, **5ax**, **5au**) did not yield compounds with neuroprotective ability as determined by the MAP2 fluorescence assay.

*Heterene derivatives.* Pyridyl-substituted triazoles **5an-5ap** included the two most promising compounds. **5ap** (with a 3'-substitution pattern) and **5ao** (4'-substitution) both demonstrated neuroprotection, reducing kainate induced loss in MAP2, and also preventing motor neuron damage in vitro, with **5ap** rescuing kainate-induced reduction in calcium flux in cortical neurons.: We note both **5ao** and **5ap** are more active than 2'-pyridyl **5an** which demonstrated increased MAP2 levels but not at a high-enough activity to carry forward into the secondary screen. The 2'-Thiophenyl triazole **5aq** was protective in reducing kainate-induced MAP2 loss but, the 3'-isomer, **5ar** had no positive activity.

*Electron-rich aryl derivatives.* Compound **5r**, protected MAP2 fluorescence from kainate treatment, has the addition of an amino group at position 6. Similar derivatives include compounds **5p** and **5q** which have the amino group at position 4 and 5 respectively, but neither afforded significant neuroprotection, with neuron. Compounds **5c**, **5d** and **5e** had the inclusion of a methyl group onto the phenyl ring, at positions 4, 5 and 6 respectively, but none exhibited neuroprotection.

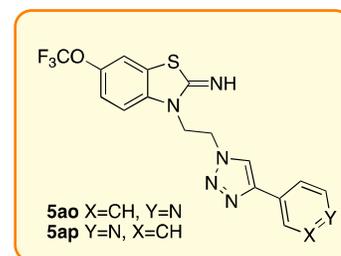
*Electron-deficient aryl derivatives.* *para*-(Bromo)phenyl triazole **5w** prevented kainate-induced MAP2 loss in the primary MAP2 screen but was ineffective on motor neurons, Its *ortho*-isomer **5x** had no effect. Chlorination was uniformly unsuccessful: compounds **5t-v** exhibited no neuroprotection activity. Mono- (compounds **5y-5aa**), di- (**5ab-5ad**) or trifluorinated (**5ae**) compounds exhibited no neuroprotection activity, while *para*-(fluoro)phenyl compound **5z** induced increased MAP2 fluorescence but not to an extent

great enough to merit further screening. (Trifluoromethyl)phenyl triazoles (**5af-5ah**) did not exhibit protection. The *para*-(Carboxy)phenyl triazole **5ak**, increased, but the corresponding ester **5aj**, decreased MAP2 fluorescence compared to kainate alone, and demonstrated toxicity in its own right (results not shown).

These data offer the promise of using entirely novel chemical matter for application in ALS therapy, either as drug-like substances or chemical probes. The next phase in this research will focus on structural modification and SAR of the small molecules with riluzole-like activity (compounds **5ao** and **5ap**), and on the use of less active compounds (compounds **5w** and **5ak**) as start-points for design and implementation of chemical probes (such as 'Click' reagents).

## Summary

Using a riluzole-triazole hybrid library **5a-5aab** and a novel motor neuron screen, we have identified seven new triazoles with neuroprotective activity greater than the parent, riluzole. Of this subset our data shows that the pyridyl-substituted compounds **5ao** and **5ap** are the most promising, with neuroprotective properties greater than riluzole in two independent in vitro assays on primary neurons. These represent promising chemical start-points for mechanistic studies. We are currently engaged in the design and delivery of chemical probes based on these structures, and in the application of this novel chemical matter to further studies of ALS and related conditions.



## ASSOCIATED CONTENT

### Supporting Information

Experimental details for preparation of compounds **5a-5aab**, including characterization data (PDF).

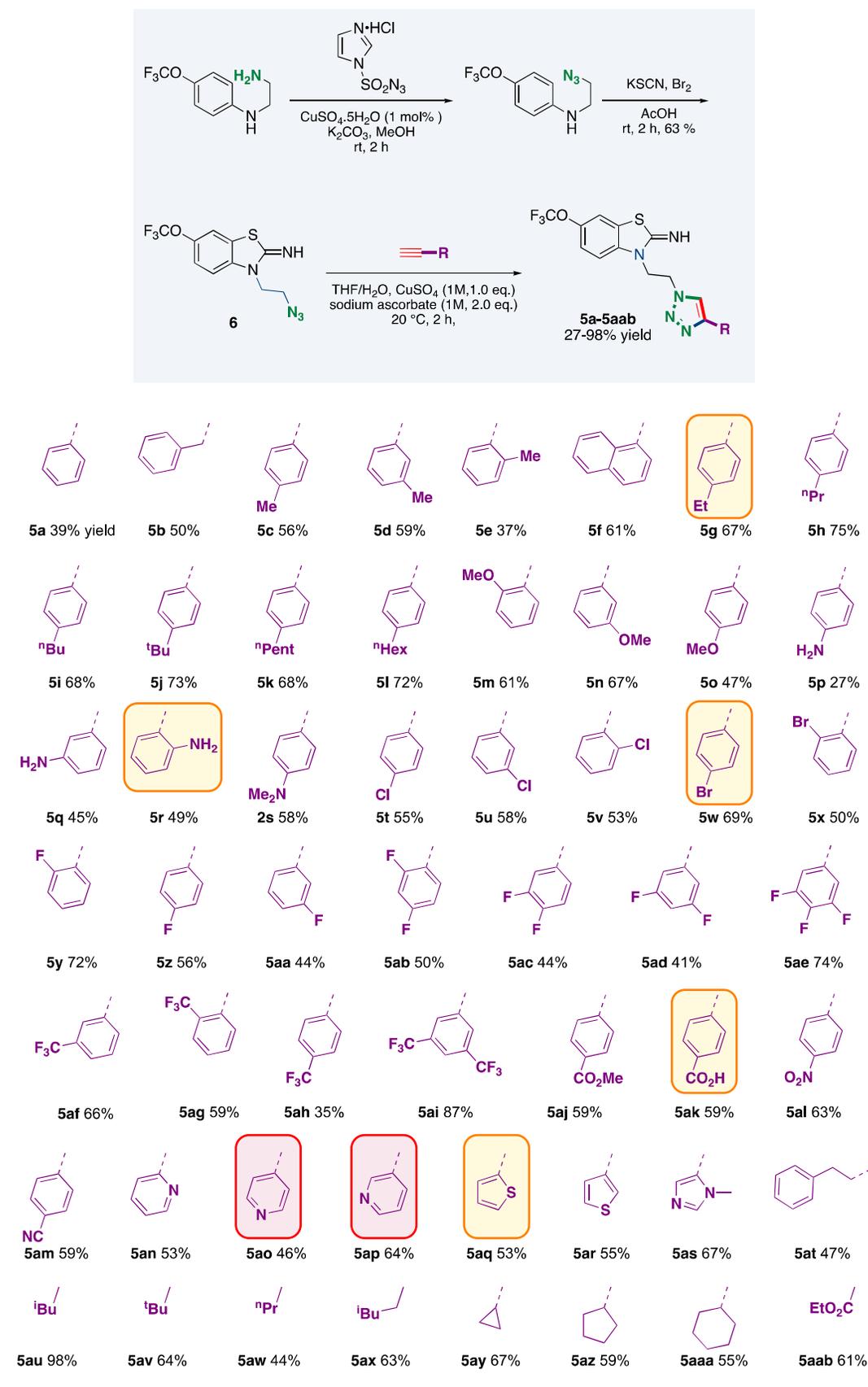
## ACKNOWLEDGMENT

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**Lay summary.** Motor neuron diseases, such as ALS – Lou Gehrig's disease – are very difficult to treat: this is partly due to the lack of understanding of the way the diseases works, and also due to the lack of new medicines which are effective. This project is focused on making new drug-like molecules, hoped to be effective in providing protection to neurons similar to those damaged in ALS. The study identified two new structures which could protect neurons; these compounds are therefore promising new tools in the treatment and understanding of ALS.

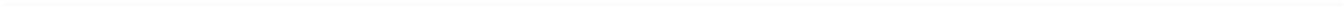
## References

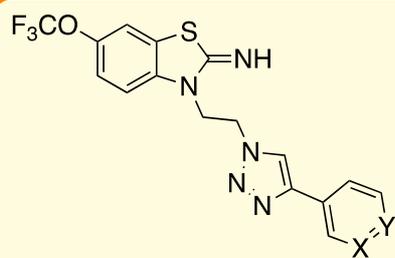
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**Table 1.** Preparation of triazolyl library **5a-5aab** (active compounds highlighted)

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*Similar  
neuroprotective  
activity to riluzole  
(X/Y=CH/N)*