Targeting Protein Kinase CK1δ with Riluzole: Could It Be One of the Possible Missing Bricks to Interpret Its Effect in the Treatment of ALS from a Molecular Point of View?

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Riluzole, approved by the US Food and Drug Administration (FDA) in 1995, is the most widespread oral treatment for the fatal neurodegenerative disorder amyotrophic lateral sclerosis (ALS). The drug, whose mechanism of action is still obscure, mitigates progression of the illness, but unfortunately with only limited improvements. Herein we report the first demonstration, using a combination of computational and in vitro studies, that riluzole is an ATP-competitive inhibitor of the protein kinase CK1 isoform δ, with an IC_{50} value of 16.1 μM. This allows us to rewrite its possible molecular mechanism of action in the treatment of ALS. The inhibition of CK1δ catalytic activity indeed links the two main pathological hallmarks of ALS: transactive response DNA-binding protein of 43 kDa (TDP-43) proteinopathy and glutamate excitotoxicity, exacerbated by the loss of expression of glial excitatory amino acid transporter-2 (EAAT2).

Lou Gehrig's disease, also known as amyotrophic lateral sclerosis (ALS) or motor neuron disease (MND), is a progressive and fatal neurodegenerative disorder that involves both upper and lower motor neurons in the brain and spinal cord.[1] Clinical symptoms reported in affected patients range from muscles weakening to spasticity, resulting in difficulty in movements, poor functional capacity and, in the final stages of the disease, respiratory failure.[2] Prognosis is grim, life expectancy rarely exceeds five years, and available drugs only mitigate progression of the illness.[3] Similar to what is reported in other neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, a pathological hallmark of ALS is the cytoplasmic aggregation of misfolded, ubiquitinated, and hyperphosphorylated proteins.[4] Transactive response DNA-binding protein of 43 kDa (TDP-43) was identified as the main component of neuronal and glial cytoplasmic inclusions, detected in grey matter urea fractions of ALS patients.[4] TDP-43 is a nuclear DNA/RNA binding protein that controls RNA transcription, maturation, and stability. Under pathological conditions, the localization and consequent function of TDP-43 is deeply altered: from the original nuclear compartmentalization the protein is sequestered in hyperphosphorylated cytoplasmic aggregates. TDP-43-mediated degeneration of motor neurons can therefore be the consequence of a nuclear "loss of function" mechanism, or can derive from a "gain of novel toxicity" in cytoplasmic inclusions; the scientific community is still debating that, but the former hypothesis is currently more accredited.[5,6] Abnormal phosphorylation plays a crucial role in TDP-43 proteinopathy: immunohistochemical analysis has identified Ser379, Ser403/4, and Ser409/410 as key conserved phosphorylated residues in pathological tissues.[7,8] Among the various protein kinases that share the ability to phosphorylate TDP-43, it has been demonstrated in both in vitro and in vivo experiments that protein kinase CK1 isoform δ (CK1δ) is the only kinase able to promote mislocalization and cytoplasmic accumulation.[8]

Despite the central role mediated by TDP-43 in motor neuron death, over the last decades other mechanisms have been proposed to explain the complex pathological progression of ALS. The discovery of an increased level of the neurotransmitter glutamate in cerebrospinal fluid of patients with sporadic nonfamiliar ALS corroborate the excitotoxic hypothesis.[9] Glutamate is the main excitatory amino acid in the brain; glial cells efficiently prevent its accumulation and toxicity in the synaptic cleft of neurons through specific uptake systems. Abnormal concentrations of glutamate would indeed disrupt the ionic equilibrium inside cells, promoting excessive entry of extracellular calcium and sodium. If the depolarization is persistent, this can be one of the reasons behind motor neuron death.[9,10] To date, there are only two drugs approved for ALS: riluzole and edaravone. Neither of these two treatments allows healing from the pathological condition; they only delay the need for assisted ventilation—and ultimately death—by several months.[11]

Riluzole, a benzothiazole compound (Figure 1), was initially investigated in the 1980s as a free radical scavenger for stroke therapy. It was then approved in 1995 for the treatment of ALS. The mechanisms of action of riluzole remain unclear, and without specific knowledge of the molecular target(s), any structure-based drug design (SBDD) approach to improve its modest activity would be impossible.[12] Neuropharmacological studies in the past years have unveiled the ability of riluzole to depress glutamatergic communication in the brain; however, none of these studies have been able to demonstrate a direct

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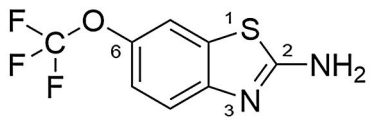


Figure 1. Structure of 6-(trifluoromethoxy)-1,3-benzothiazole-2-amine, commercially known as riluzole.

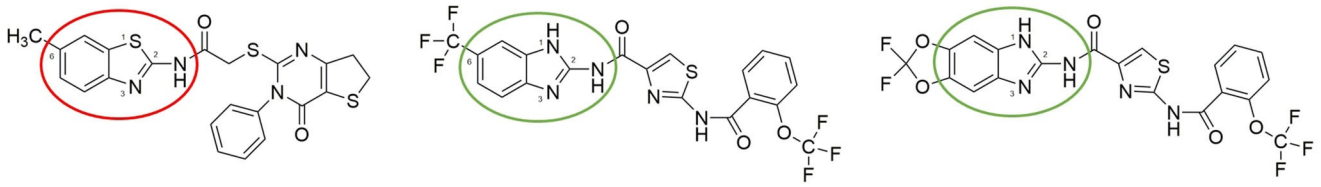
interaction between the drug and ionotropic/metabotropic glutamate receptors or transporters.[10,13] In addition, traditional antiglutamatergic molecules showed no therapeutic effect in ALS models; all these findings suggest that riluzole modulation of glutamate signaling may be indirect, through a third-party molecular actor.

In this study, we initially hypothesize, through computational studies, and later experimentally demonstrate, the inhibitory mechanism of action of riluzole toward the target CK1δ.

CK1δ, the protein responsible for TDP-43 pathological hyperphosphorylation, is a Ser/Thr protein kinase ubiquitously expressed in eukaryotic organisms, mainly in the central nervous system (CNS).[14] Different families of potent CK1δ inhibitors were developed during the last decades, but until now, none of them have reached clinical trials due to a low selectivity profile and/or poor CNS permeation.[15] Nowadays, 18 CK1δ crystallographic structures are present in the RCSB Protein Data Bank (PDB), 15 of which are co-crystal structures with an ATP-competitive inhibitor (all data are provided in the Supporting Information).

Our computational work started with a conventional visual inspection of all co-crystallized ligands followed by scaffold clustering based on different similarity metrics such as MACCS-driven Tanimoto and Dice indexes. Curiously, three ATP-competitive inhibitors belong to the benzothiazole and benzimidazole families: in particular, as shown in Figure 2, the compound 9XK (PDB ID: 5OKT) is a 2-aminobenzothiazole, whereas compounds 37J (PDB ID: 4TWC) and 386 (PDB ID: 4TW9) are 2-aminobenzimidazoles.[16–18] Looking at the ATP-binding site, both benzothiazole and benzimidazole scaffolds are anchored to the hinge region of the protein kinase through a conserved hydrogen bond network, similar to what was also observed for the ATP binding mode. Furthermore, the benzothiazole scaffold has been extensively investigated as a starting point for computer-aided design and for the subsequent synthesis of potent CK1δ inhibitors, for which structural data are not available.[19,20] Intriguingly, riluzole also belongs to the 2-aminobenzothiazole family and, theoretically, it could itself be an ATP-competitive inhibitor of CK1δ. Currently, no information on this is available. To preliminarily support this hypothesis, a molecular docking study was performed, to determine if the mode of interaction of riluzole is consistent with that observed for the three crystallographic inhibitors described above. Figure 3A shows the energetically more stable binding mode for riluzole in the CK1δ catalytic site. The orientation of the 2-aminobenzothiazole scaffold is nicely superimposable with the crystallographic binding mode of the aforementioned kinase inhibitors (see the Supporting Information). In particular, Leu85 seems to play a fundamental role in binding, stabilizing riluzole to the kinase hinge region with a double hydrogen bond interaction: the first one between the carbonyl group of the backbone and the amino group at the 2-position of riluzole, and the second between the NH group of the backbone and the nitrogen atom at the 3-position of riluzole. Moreover, the riluzole trifluoromethoxy group occupies a hydrophobic pocket located near Met82, the crucial "gatekeeper residue" of the kinase. Indeed, most of the co-crystallized CK1δ inhibitors are characterized by the presence of halogen atoms at the same position of the trifluoromethoxy group of riluzole, supporting the importance of this substituent for binding recognition. Using a heat map representation, Figure 3B quantitatively depicts the electrostatic and hydrophobic contributions of ligand binding mediated by all residues that compose the ATP-binding cavity.

Regarding the electrostatic contributions, the fundamental role of Leu85 is once again underscored by the corresponding intense blue band. On the other hand, observing the hydrophobic contacts, several residues such as Ile23, Tyr56, Leu85, and Ile148, contribute to the overall stabilization of the ligand–kinase complex. This aspect is common to all crystallographic benzothiazole/benzimidazole CK1δ inhibitors for which, in addition to a conserved pair of electrostatic interactions, the hydrophobic contribution seems to be the predominant driving force of ligand binding (more details are given in the Supporting Information).



9XK – 5OKT		37J – 4TWC		386 – 4TW9	
Tanimoto Index	Dice Index	Tanimoto Index	Dice Index	Tanimoto Index	Dice Index
0.63	0.78	0.66	0.79	0.63	0.77

Figure 2. Structures of CK1δ crystallographic inhibitors 9XK, 37J, and 386 characterized by the highest values of molecular similarity with respect to riluzole; benzothiazole and benzimidazole scaffolds responsible for protein kinase recognition are indicated with red and green circles.

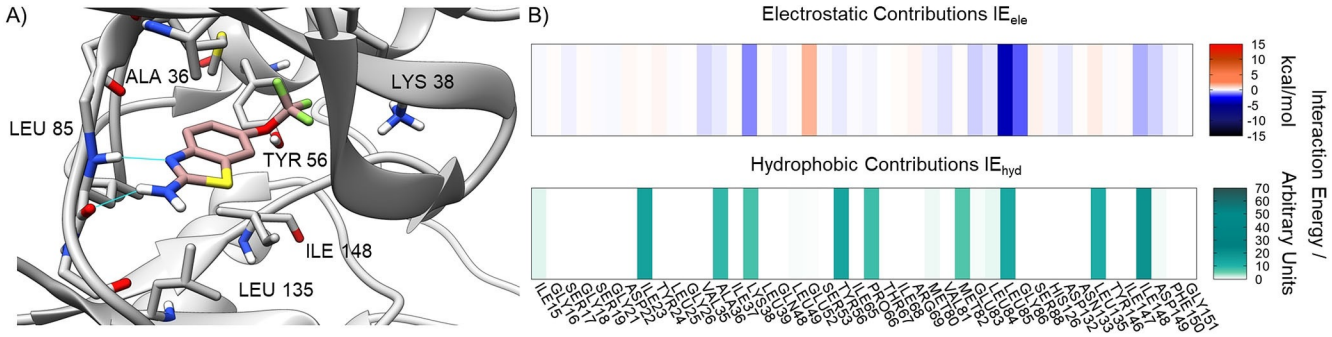


Figure 3. A) Molecular docking predicted pose for riluzole in the CK1δ ATP binding site (PDB ID: 5OKT); residues involved in molecular recognition are labeled, while hydrogen bonds between the drug and kinase hinge region are depicted by light blue lines. B) Interaction energy fingerprints that summarize, using a heat map, the electrostatic and hydrophobic contributions to binding mediated by each residue composing the protein binding site.

To further support the hypothesis that riluzole may be an ATP-competitive inhibitor of CK1δ, a supervised molecular dynamics (SuMD) study was carried out. SuMD is a recently developed computational technique for investigating unbiased receptor–ligand recognition pathways on the nanosecond timescale, by combining unbiased MD simulations and a *tabu*-like algorithm.[21,22] As depicted in Figure 4 A, starting from 70 Å away from the ATP binding cavity of CK1δ, in only 26 ns of SuMD simulation, it was possible to explore the entire binding recognition of riluzole to CK1δ. After 10 ns, riluzole is perfectly accommodated in the CK1δ binding site, establishing the same interaction pattern previously described by docking analysis, as confirmed by the RMSD values calculated during the SuMD trajectory. The ligand–protein interaction energy landscape of the molecular recognition pathway is depicted in Figure 4 B; it is possible to cluster three main ligand–protein populations during the SuMD trajectory. Cluster 1, energetically similar to the unbound state, describes the first contact event between riluzole and CK1δ, considerably far away from the conventional ATP binding site. Conversely, clusters 2 and 3 depict the two key steps of molecular recognition, when riluzole approaches the ATP binding site, reorienting itself to reach the final binding mode, characterized by higher energetic stability (the video in the Supporting Information shows the recognition mechanism in detail).

Surprisingly, all preliminary computational analyses converged to indicate that riluzole can bind protein kinase CK1δ, which, if demonstrated, could also allow rewriting, from a mechanistic point of view, its possible role in the treatment of ALS. To experimentally validate this hypothesis, the inhibitory activity of riluzole toward CK1δ was evaluated in vitro, through a luminescence kinase assay. Initially, two independent experiments were set up using riluzole at concentrations of 10 and 40 μM. At the higher concentration, riluzole was able to completely abolish CK1δ catalytic activity. Thus, three independent experiments at eight different concentrations of riluzole, from 100 to 0.16 μM, were carried out to precisely quantify its IC_{50} value, which was determined to be 16 μM, as reported in Figure 5. These data, although preliminary, could lay the foundation for exploring the role of riluzole in ALS treatment through its direct inhibition of CK1δ. Although the primary pathogenic role of this protein kinase in ALS is already confirmed, there remain some questions that must be clarified by future studies.[8] In particular, the causes behind the deregulated and aberrant activation of CK1δ in ALS are not known, and it also remains obscure which of the two different cellular populations of CK1δ—cytoplasmic or nuclear—are responsible for neurodegeneration.[23]

However, after more than twenty years from riluzole's approval for ALS treatment, the identification of CK1δ as a novel mo-

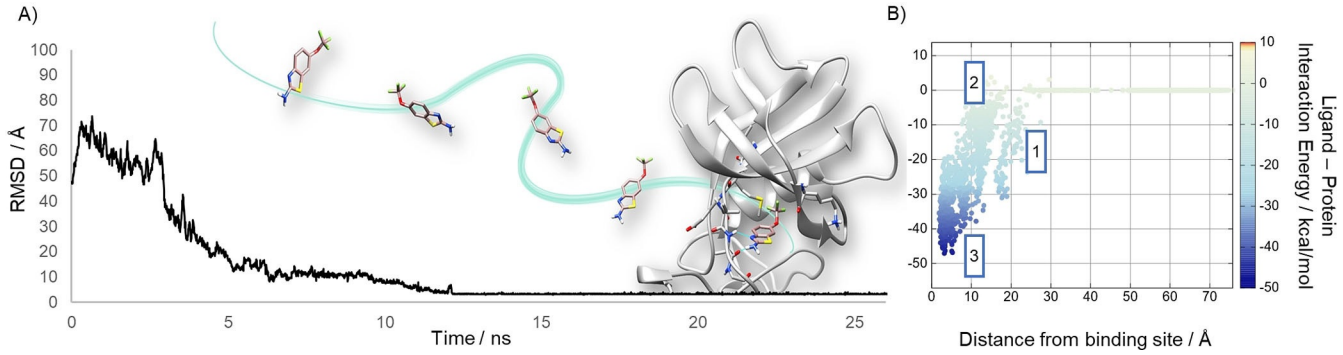


Figure 4. A) RMSD variation of riluzole's atomic coordinates during SuMD trajectories, with respect to the pose predicted by molecular docking. In the background are some snapshots of a possible molecular recognition process between riluzole and CK1δ; the final SuMD binding mode of the drug is geometrically and interactively convergent with docking results (a video of the whole SuMD trajectory is available as Supporting Information). B) Riluzole recognition energy landscape based on the potential energy contribution during SuMD trajectory; boxes 1 to 3 highlight the main clusters of energetic populations sampled during the recognition pathway.

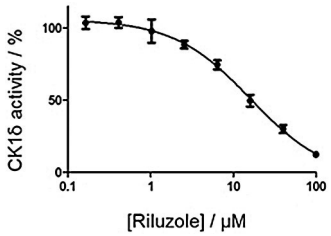


Figure 5. Concentration–inhibition curve of riluzole at human CK1δ. The obtained IC_{50} value is 16.1 μM, with a 95% confidence interval of 5.6–26.7 μM. Data were collected from three independent experiments performed in duplicate.

lecular target allows us to revisit the drug's mechanism of action, as schematized in Figure 6. In summary, riluzole inhibits the catalytic activity of CK1δ, decreases TDP-43 hyperphosphorylation, and consequently TDP-43 aggregation and cytoplasmic mislocalization, one of the principal pathological hallmarks of ALS. As reported by an independent screening of known active molecules from the LOPAC® library, riluzole was already classified as TDP-43 self-interaction inhibitor, with an unknown mechanism of action.[2] Curiously, the same work found that the concentration at which riluzole exerts its maximum effect is around 10 μM, in agreement with the IC_{50} value determined in vitro toward CK1δ. In parallel, the pathological loss of TDP-43 nuclear localization in ALS results in the progressive disruption of normal mRNA maturation. In particular, it was experimentally found in samples obtained from human brains, that the levels of mRNA transcripts for glial excitatory amino acid transporter-2 (EAAT2) are significantly lower in TDP-43 proteinopathy-affected patients relative to samples taken from healthy people.[24]

EAAT2 is normally expressed in glial cells, and it is responsible in synaptic boutons for almost 90% of glutamate reuptake, thereby preventing its accumulation and toxicity. CK1δ inhibitors are able to restore the correct nuclear localization of TDP-43, and thus riluzole itself modulating CK1δ activity could ensure the physiological processing and maturation of specific mRNA transcripts, mediated by TDP-43.[25] This hypothesis found an experimental confirmation in two independent studies: in the first, riluzole showed the ability to reverse cocaine-induced suppression of the glutamate transporter EAAT2 in the nucleus accumbens (NAc), and coherently, in the second study, long-term riluzole administration rescued EAAT2 mRNA levels and protein expression in the hippocampus.[26,27] Moreover, in a murine model, depletion of EAAT2 promoted the progressive impairment of glutamate reuptake, with consequent death of motor neurons, muscular weakness, and paralysis conditions similar to those of ALS.[28]

All this evidence allows us to speculate that riluzole, through the inhibition of CK1δ, ensures the physiological nuclear compartmentalization of TDP-43, which guarantees the correct maturation of EAAT2 mRNA transcripts. Therefore, the normal expression of the glutamate transporter on astrocytes prevents and protects motor neurons from excitotoxity. Interestingly, in this proposed mechanism, the role of glial cells in the pathological onset of ALS takes on greater importance, if compared with the classical motor-neuron-centric hypothesis, and this is coherent with Sloan and Barres' research, which has clarified the role of astrocytes in TDP-43 proteinopathy.[29]

In the present work, it has been demonstrated for the first time, through both computational and in vitro studies, that riluzole is an ATP-competitive inhibitor of the protein kinase CK1δ. This clarifies its possible therapeutic role in the treatment of ALS, bridging the two main pathological hallmarks of the disease: TDP-43 aggregation and excitotoxicity. Further studies are still ongoing in our research group to confirm the proposed mechanism of action and to elucidate if more potent and CNS-permeable CK1δ inhibitors are able to replicate and improve the therapeutic profile of riluzole.

Acknowledgements

The Molecular Modeling Section (MMS) laboratory is very grateful to Chemical Computing Group, OpenEye, and Acellera for the scientific and technical partnerships. The MMS lab gratefully acknowledges the support of NVIDIA Corporation with the donation

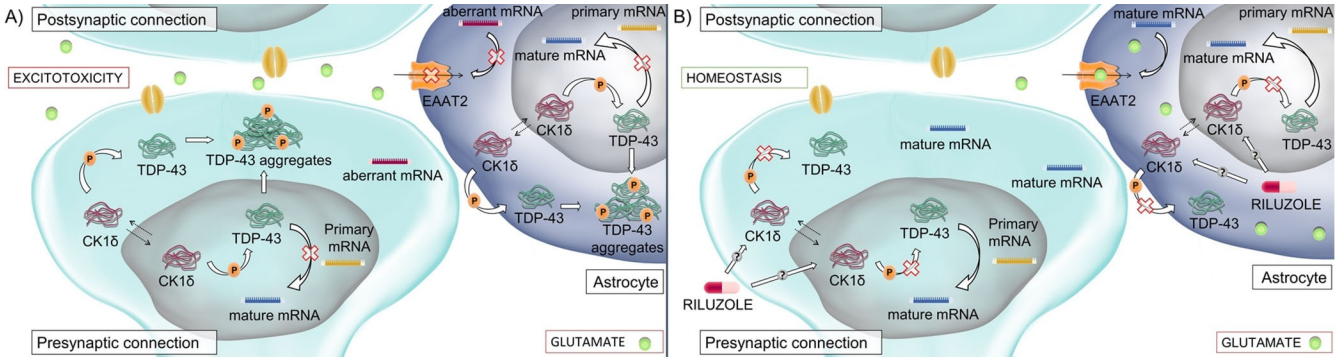


Figure 6. A) Excitotoxicity conditions in the synaptic cleft: because it is not known which population of CK1δ is responsible for TDP-43 hyperphosphorylation and mislocalization, both nuclear and cytoplasmic populations are shown. Loss of TDP-43 nuclear localization causes aberrant mRNA maturation and thus the lack of expression of EAAT2 on the astrocyte, with consequent glutamate accumulation and toxicity. B) Riluzole, in our proposed mechanism of action, inhibits CK1δ both in neurons and in glial cells, preventing TDP-43 proteinopathy, thereby allowing the physiological expression of EAAT2 and indeed glutamate homeostasis.

of the Titan Xp GPU used for this research. The authors thank Alessia Vitale (University of Trieste) for her help in kinase activity testing.

Conflict of interest

The authors declare no conflict of interest.

Keywords: amyotrophic lateral sclerosis · mechanism of action · protein kinase CK1δ · riluzole · TDP-43

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Accepted manuscript online: O ctober 25, 2018