Multitarget Hybrid Fasudil Derivatives as a New Approach to the Potential Treatment of Amyotrophic Lateral Sclerosis

Olmo Martín-Cámara, Marina Arribas, Geoffrey Wells, Marcos Morales-Tenorio, Ángeles Martín-Requero, Gracia Porras, Ana Martínez, Giorgio Giorgi, Pilar López-Alvarado, Isabel Lastres-Becker, and J. Carlos Menéndez

1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a degenerative disease that leads to the destruction of neuromuscular junctions of the first and second motoneurons (MNs), thereby causing progressive muscle weakness and atrophy accompanied by exaggerated tendon reflexes. About 35% of patients with ALS suffer behavioral or cognitive impairment, with an additional 15% having frontotemporal dementia. After a few years, this paralysis generally becomes lethal due to overall respiratory failure. The prevalence of ALS ranges from 2 to 5 cases per 100,000 and occurs sporadically (sALS) or in a familial form (fALS). The best-known cause of ALS, responsible for one-fifth of fALS cases, is a mutation in the gene encoding superoxide dismutase 1 (SOD1), an enzyme abundant in the cytoplasm and mitochondria of virtually all cell types. Although this mutation was the first to be identified, more recently, several pathogenic mutations related to ALS have been found, such as those in the TDP-43, FUS, and C9ORF72 proteins. Such mutations can lead to the formation of toxic aggregates, which increase oxidative stress defense NRF2 expression, are associated with a delayed disease onset of ALS. These results suggested that variations in NEF2L2, which encodes the master regulator of oxidative stress defense NRF2, may affect sALS progression.

Despite multiple clinical trials to date, only the glutamate release inhibitor riluzole, the free-radical scavenger edaravone, and the tyrosine kinase inhibitor masitinib have been approved for the treatment of ALS, in the latter case for compassionate use. The effect of these drugs is very modest and prolongs the survival of patients by only a few months. The mechanisms of ALS pathogenesis involve multiple factors that include protein aggregation, oxidative stress, mitochondrial dysfunction, excitotoxicity, disturbance of selective autophagy pathways, degenerative processes related to neuron–glia interactions, alterations in RNA metabolism, cytoskeletal defects, and apoptosis. This suggests that, rather than addressing a single target, treatments of this disorder should be directed to different molecular pathways through a multidrug combination therapy.

A common feature shared by ALS and several additional neurodegenerative disorders that affect voluntary muscle movement is the alteration of the activity of Rho GTPase, a protein that forms part of the Rho-ROCK signaling pathway and regulates the formation of the actin cytoskeleton in nerve cells. The inhibition of Rho kinases (ROCK), a family of serine/threonine kinases, using small molecule inhibitors, such as fasudil or Y-27632, may not only improve the regenerative
response in the injured central nervous system (CNS) but also improve neuronal survival,\(^\text{15-17}\) including the promotion of neuromuscular junction maturation.\(^\text{18}\) For this reason, the U.S. Food and Drug Administration (FDA) has allowed fasudil to be tested in clinical trials for ALS\(^\text{19}\) and accepted its compassionate use in ALS patients.\(^\text{20}\)

On the other hand, the role of oxidative stress, inflammation, and mitochondrial dysfunctions as important pathogenic mechanisms in ALS is well-established. Several antioxidant molecules and detoxifying enzymes are implicated in the defense against oxidative stress. The most important of these mechanisms is orchestrated by NRF2 (nuclear factor erythroid 2-related factor 2), the master regulator of cellular redox homeostasis.\(^\text{21}\) In nonstressed conditions, the N-terminal domain of the cap’n’collar homology (ECH)-associated protein 1 (KEAP1) presents NRF2 for ubiquitination by cullin 3 and RING-box protein1 (CUL3/RBX1)\(^\text{22}\) and subsequent degradation by the proteasome. In response to oxidative or electrophilic stressors, KEAP1 loses its ability to repress NRF2 due to modification of critical cysteines, leading to NRF2 stabilization and activation of its transcriptional activity.\(^\text{23,24}\) An alternative mechanism of regulation of NRF2 stability involves the phosphorylation of its Neh6 domain mediated by glycogen synthase kinase 3 (GSK-3), which initiates the recruitment of the \(\beta\)-transducin repeat-containing protein (\(\beta\)-TrCP) and facilitates the interaction between NRF2 and the CUL1/RBX1 complex for ubiquitin-proteasome degradation of NRF2.\(^\text{25}\) In the nucleus, NRF2 dimerizes mainly with the cognate bZip partners MAF G, K, and F and then binds to the antioxidant response element (ARE) activating the transcription of cytoprotective genes including several antioxidant and anti-inflammatory enzymes, which makes this pathway an increasingly important target in neurodegenerative diseases.\(^\text{26}\) In relation to ALS, it has been shown that NRF2 mRNA and protein levels were reduced in ALS patients relative to control tissues,\(^\text{27,28}\) although NRF2 target genes were not analyzed. Recent studies performed in our laboratory in ALS patient-derived lymphoblasts, which recapitulate features of affected MNs,\(^\text{29}\) clearly demonstrated that NRF2 activity appears to be differentially regulated in sALS or SOD1-ALS.\(^\text{30}\) These data indicate that pharmacological modulation of NRF2 as a therapeutic strategy for ALS should be personalized according to the molecular differences displayed by the patient. Indeed, NRF2-activating compounds have demonstrated therapeutic efficacy in SOD1 mouse models of ALS.\(^\text{31}\)

In the context of the multitarget-directed ligand (MTDL) approach, i.e., the purposeful design of small molecules able to inhibit several pathological mechanisms, we have focused on preventing damage triggered by oxidative stress through NRF2 activation while simultaneously avoiding the pathological consequences of ROCK overphosphorylation using a polypharmacological approach. To this end, we have hybridized the ROCK inhibitor fasudil with two natural products (caffeic and ferulic acids) capable of inducing the activation of NRF2 that also have radical scavenging properties due to their phenolic nature (Figure 1).

While multitarget approaches are becoming increasingly popular in drug discovery against multifactorial diseases,\(^\text{32,33}\) their application to ALS therapy has received little attention. The peptidic drug alirinetide (GM604, GM6, L-phenylalanyl-L-seryl-N\(^5\)-(diaminomethylene)-L-ornithyl-L-tyrosyl-L-alanyl-N\(^3\)-(diaminomethylene)-L-ornithine), which was granted fast track status by the FDA and orphan drug designation by the EMA and has undergone phase II clinical trials for the treatment of ALS,\(^\text{33}\) is believed to promote neuron survival via a multitargeted regulation of developmental pathways,\(^\text{34}\) although it was not designed using the MTDL paradigm. Regarding small molecules, the multitarget iron chelator VAR10303 has shown beneficial effects on ALS mice.\(^\text{35}\) In this context, we describe here the design and synthesis of a small library of fasudil-ferulic/caffeic hybrid compounds and their characterization as an NRF2 signaling inducer and their therapeutic potential, especially for ALS patients with a SOD1 mutation.

2. RESULTS AND DISCUSSION

2.1. Compound Design. The fasudil-based hybrid compounds studied here are shown in Figure 2. Together with the cinnamic acid derivatives, we also planned the preparation of the corresponding dihydro derivatives, to establish the relevance of the double bond on NRF2 induction. To our knowledge, there has been only one precedent of a multitarget drug designed from fasudil, in which it was coupled to an antioxidant (lipoic acid); this compound proved to be less cytotoxic than other fasudil derivatives due to the protective effect of the second structural fragment.\(^\text{36}\)
First, computational ADME studies were carried out using SwissADME. For all compounds, a high gastrointestinal absorption, important for oral bioavailability, was predicted. Moreover, no compound showed violations of Lipinski’s rule of five. The details of this study are shown in Table S1 (see the Supporting Information). A single PAINS alert appeared in some of the compounds due to the presence of catechol moieties. In order to discard false positives by nonspecific target binding, we studied the activity of compound 1d as an inhibitor of the kinase GSK-3β and also its ability to reduce aberrant TDP-43 phosphorylation or TDP-43 expression in lymphoblasts from ALS patients (see the details in the Supporting Information, Figure S1). The negative results of all these experiments allow us to discard indiscriminate binding of 1d to biological targets. On the other hand, a complementary study performed with ADMETLab 2.0 gave less favorable results in terms of oral absorption and raised some toxicity concerns that will need to be addressed in future optimization efforts (Table S2).

We also assessed computationally whether our planned structural manipulation of fasudil would maintain affinity for the ROCK enzyme. Two main isoforms of the enzyme (ROCK1 and ROCK2) are known, with ROCK2 being the predominant form expressed in smooth muscles and the brain. For this reason, a crystal of the ROCK2 isoform (PDB 4WOT) was selected to perform the docking studies of compounds 1. In the case of fasudil, which was studied in the first place in order to validate the docking protocol, all the critical interactions previously established by X-ray crystallography, i.e., a hydrogen bond with Met172 of the hinge region, and nonpolar interactions with some other residues in the hydrophobic pocket (Leu221, Ala231, Val106, and Met169) were located (Figure 4A). In the case of compounds 1, similar interactions were found, together with some distant interactions of the catechol moiety with the protein (Leu123 and Phe136 residues). The binding energies of the whole family of compounds 1 were studied with Autodock Vina. As shown in Table 1, most of the compounds show similar or higher binding energies than the reference compound fasudil. Moreover, a careful examination of the energies reveals that the dihydro ligands (1c, 1d, 1g, and 1h) show in all cases lower energies than the corresponding unsaturated compounds (1a, 1b, 1e, and 1f). This behavior can be explained due to an increase in the degrees of freedom in the side chain of the dihydro ligands, which allows the catechol moiety to accommodate better to the distant region (Leu123 and Phe136).

Table 1. Free-Energy Estimation (kcal/mol) for the Complexes Formed by Compounds 1 and ROCK2

| compound | energy (kcal/mol) |
|----------|-------------------|
| fasudil  | 8.00              |
| 1a       | 7.70              |
| 1b       | 8.80              |
| 1c       | 8.20              |
| 1d       | 10.30             |
| 1e       | 7.80              |
| 1f       | 8.10              |
| 1g       | 8.50              |
| 1h       | 9.37              |

To further analyze the stability of the system and the different binding energies, molecular dynamics simulations (10 ns) and subsequent metadynamics were performed on fasudil and compound 1d (Figure 3). Both fasudil and 1d gave stable complexes with the enzyme along the simulation trajectory. To determine the stability of the complexes of fasudil or 1d with the enzyme along the simulation trajectory, we calculated the root-mean-square deviation (RMSD) of the ligand structure compared with the values obtained after energy minimization for each snapshot of the simulation. In the case of fasudil, the initial conformation remained very stable, with an RMSD value of 1.77 ± 0.35 Å. In the case of 1d, the compound showed an RMSD value of 2.18 ± 0.24 Å, which is less stable than fasudil but still considered stable. We also determined the stability of the hydrogen bond of the ligand with the hinge region by measuring the evolution of the distance between the isoquinoline nitrogen and the backbone nitrogen-bonded hydrogen of Met172 (d[N(ligand)–H(Met172)]) for each snapshot of the simulation. The average hydrogen bonding distance for fasudil was 2.02 ± 0.17 Å, and it remained below 2.5 Å nearly all the time (98.8%). On the other hand, d[N(1d)–H(Met172)] was 2.19 ± 0.56 Å on the average, and it remained below 2.5 Å for 90.8% of the time.

The binding energy for each pose was calculated over 200 snapshots of the complex from the final 2 ns of simulation. The free energy of binding was obtained by using the molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) approach, which calculates the final energy from terms corresponding to potential energy in vacuum (van der Waals energy and electrostatic energy) and solvation energies (including polar and nonpolar terms). The results of these calculations are given in Table 2.

After the assessment of the computational stability of the complex of compound 1d and ROCK2, we concluded that our planned introduction of the cinnamic/dihydrocinnamic side chains is in principle compatible with ROCK2 inhibition.

2.2. Synthesis. The starting materials 2 were prepared from isoquinoline-5-sulfonic acid and piperazine or homopiperazine using a literature method. Their treatment with cinnamic (R = H) or ferulic (R = Me) acid using a combination of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI) and 1-hydroxybenzotriazole (HOBt) as coupling reagents afforded compounds 1a,b,e,f in moderate yields. A similar treatment with dihydrocaffeic and dihydroferulic acid furnished phenylpropionamide derivatives 1c,d,g,h (Scheme 1).

2.3. Rho Kinase Inhibition Studies. Because ROCK2 predominates in the human brain, we focused on the ability of compounds 1 to inhibit this isoform of the enzyme. As summarized in Table 3, we measured IC50 values for all compounds and also for fasudil (2b), and these data led to the conclusion that piperazinamide derivatives are generally less active as ROCK2 inhibitors than their homopiperazine counterparts (1a > 1b, 1c > 1d, and 1e > 1f), catechols are more active than O-methylcatechols (1a > 1e, 1b > 1f, 1c > 1g, and 1d > 1h), and the double bond generally favors ROCK2 inhibition (1a < 1c, 1b < 1d, and 1e > 1f). From these studies, compounds 1a, 1c, and 1d were shown to inhibit ROCK2 with a potency similar to the reference compound. These experimental data are in good agreement with the computational studies, which predict a similar or higher free energy for the binding of catechol derivatives in comparison to their O-methyl derivatives and also a better binding of the dihydro derivatives.
2.4. Radical Scavenging Capacity. As mentioned in the Introduction, oxidative stress is an important factor in the progression of ALS, and thus, we tested the potential antioxidant effect of our compounds as direct ROS scavengers. DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) is a stable free radical that is reduced in the presence of antioxidant molecules, giving a colorless solution. The DPPH reduction antioxidant assay was employed to study compounds 1, finding that, as expected in view of their phenol functional groups, they have a good radical scavenging capacity. The DPPH reducing activity of our compounds was similar to those of caffeic and ferulic acids, which were used as references for the assay. The catechol derivatives 1a−d were more effective as free-radical scavengers than their O-methyl counterparts 1e−h (Table 4).

2.5. Experimental ADME Studies. In order to extend the above-mentioned computational ADME profile, we have studied experimentally some in vitro experimental properties of compound 1d, which was our best candidate for further optimization, as shown in subsequent sections. Most of the therapeutic compounds are biotransformed in the liver tissue, and therefore, liver microsomal fractions are widely used to study the in vitro metabolic stability in drug discovery phases. Compound 1d was incubated with human microsomes, using as a control for the assay verapamil, a widely used drug with a
well-known metabolism. The results obtained indicate that 1d presents a better metabolic behavior than verapamil (Table 5), with a higher half-life ($t_{1/2}$) and lower intrinsic clearance (CLint), which correspond to a longer time of compound exposure in vivo.

**Table 3. ROCK2 Inhibition by Compounds 1 and the Reference Compound Fasudil, Expressed as IC$_{50}$ Values ($\mu$M)**

| compound | IC$_{50}$ ($\mu$M) | SD |
|----------|-------------------|----|
| fasudil  | 0.37              | 0.08 |
| 1a       | 0.79              | 0.25 |
| 1b       | 2.13              | 0.45 |
| 1c       | 0.32              | 0.09 |
| 1d       | 0.73              | 0.13 |
| 1e       | 2.33              | 0.78 |
| 1f       | 2.61              | 0.28 |
| 1g       | >10               | n/a |
| 1h       | 0.90              | 0.11 |

**Table 4. Radical Scavenging Activity of Compounds 1, Expressed as EC$_{50}$ Values (DPPH Method)$^{a}$**

| compound | EC$_{50}$ ($\mu$M) | SD |
|----------|-------------------|----|
| caffeic acid | 12.13           | 1.03 |
| 1a       | 9.93              | 1.18 |
| 1b       | 10.02             | 0.96 |
| 1c       | 10.11             | 0.69 |
| 1d       | 9.68              | 0.66 |
| ferulic acid | 22.83           | 0.69 |
| 1e       | 6.42              | 0.47 |
| 1f       | 9.61              | 0.65 |
| 1g       | 10.30             | 0.63 |
| 1h       | 12.00             | 0.99 |

$^{a}$Caffeic and ferulic acids were employed as references.

**Table 5. Stability of Compound 1d in Human Liver Microsomes**

| compound | $t_{1/2}$ (min) | CLint$^{a}$ (mL/min/mg protein) |
|----------|----------------|---------------------------------|
| 1d       | 35.7           | 15.3                            |
| verapamil | 20.2           | 26.9                            |

$^{a}$CLint, intrinsic clearance.$^{44}$

Protein binding is another important parameter related to the ADME profile. In particular, the binding of drugs to serum albumin is the key to their distribution in the body, either by influencing the effective concentration of the drug at its site of action, since only an unbound drug is able to reach its target, or by changing the rate at which the drug is eliminated by interference with its glomerular filtration. For this reason, we studied the binding of compound 1d to human serum albumin (HSA). It is well-known that the fluorescence quenching effect is an indirect method for studying the binding of small molecules to proteins such as HSA.$^{45,46}$ and Figure 4 shows that the addition of increasing amounts of compound 1d to a solution of human serum albumin (HSA) diminished the intensity of the fluorescence of the protein ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 337$ nm). The quenching effect is evident at lower ratios, from 0/1 to 1/1 (1d/HSA), but at higher molar ratios, this effect diminished, suggesting that the system becomes saturated. In order to determine the nature of the quenching effect, the Stern–Volmer equation was applied, showing a linear behavior until the molar ratio reaches 1/1 (Q/HSA). From that point, the representation is not linear, which also suggests saturation effects. These data prove that compound 1d binds to HSA generating a quenching effect defined by the constant $K_{SV} = 1.68 \times 10^{5}$ L mol$^{-1}$. In order to determine the affinity of the binding, the Scatchard equation$^{45}$ was employed, establishing that the 1d-serum albumin complex has an association constant $K_a = 7.94 \times 10^{5}$ L mol$^{-1}$. It can be concluded that 1d binds to HSA with an affinity that is in the
indicate that, with the exception of viability reduced to 61.85%). In the aggregate, these results show that all compounds have toxicities below 20% (Table 6), except for compound 1e. Our findings suggest that the concentrations used for each compound were 20 and 60 μM. In general, all compounds show toxicities below 20% (Table 6), except for compound 1e, which shows significant cytotoxic effects (cell viability reduced to 61.85%). In the aggregate, these results indicate that, with the exception of 1e, compounds 1 have a good tolerability.

Table 6. Analysis of Cell Viability of the Compounds 1, Determined by MTT Assay

| compound | 20 μM | 60 μM  |
|----------|-------|--------|
| basal    | 99.987 ± 2.023 | 92.207 ± 0.462 |
| DMF      | 90.359 ± 6.958 | 92.207 ± 0.462 |
| 1a       | 90.937 ± 1.634 | 100.680 ± 0.999 |
| 1b       | 96.873 ± 1.130 | 84.663 ± 1.827 |
| 1c       | 103.727 ± 1.204 | 104.793 ± 0.434 |
| 1d       | 100.427 ± 1.024 | 103.743 ± 0.837 |
| 1e       | 60.530 ± 2.735 | 55.927 ± 1.079 |
| 1f       | 93.913 ± 2.273 | 77.863 ± 3.899 |
| 1g       | 93.678 ± 2.202 | 87.540 ± 3.234 |
| 1h       | 99.383 ± 1.782 | 88.213 ± 2.434 |
| fasudil  | 92.070 ± 3.714 | 83.434 ± 0.715 |

Same order of magnitude as those of many well-established drugs. 27

2.6. Effect of Compounds 1 on Cell Viability. In vitro cytotoxicity testing gives essential information for safety assessment and screening and for ranking compounds. The cytotoxic effect of these compounds was evaluated against HEK293T cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The concentrations used for each compound were 20 and 60 μM. In general, all compounds show toxicities below 20% (Table 6), except for compound 1e, which shows significant cytotoxic effects (cell viability reduced to 61.85%). In the aggregate, these results indicate that, with the exception of 1e, compounds 1 have a good tolerability.

2.7. Compounds 1b, 1c, and 1d Lead to NRF2 Transcriptional Activation. To determine whether compounds 1 could activate NRF2 and induce its transcriptional activity, we used two different approaches. First, we performed a luciferase reporter assay using a promoter that contains three ARE sites in tandem (ARE-LUC). 48 Dimethyl fumarate (DMF) (20 μM), a well-known inducer of NRF2, was used as a positive control. We found a dose-dependent activation of the NRF2 reporter by 1b, 1c, and 1d (Figure 5). In the case of compound 1b, the activation of NRF2 could be due to the cytotoxic effect that we have observed previously. 49 Compound 1d, at a dose of 20 μM, had a similar effect to the positive DMF control at the same dose.

The low activity of fasudil confirmed that NRF2 reporter induction was mainly due to the presence of the cinnamic or dihydrocinnamic side chain. It is relevant to note that the three active compounds were catechol derivatives and that the double bond in the side chain does not seem to have an important role in activity since the more potent compounds 1c and 1d lack this structural feature.

Multitarget-directed ligands are generally considered to have a balanced activity profile if their potency ratio between any two targets is not higher than 10. 30 In order to study this aspect of our compounds, we have determined the values of CD (i.e., the concentration able to duplicate the response to the luciferase assay relative to basal) for those that showed activity in this regard, namely, 1b–1d. The results are shown in Table 7, together with the IC50 values for ROCK2 inhibition and the corresponding ratios. While compound 1c showed an unbalanced profile, both 1b and 1d were successful in this regard. In particular, our hit compound 1d showed a ratio of 1.4 between NRF2 induction and ROCK2 inhibition, which can be regarded as a very well-balanced profile and a good starting point for future optimization efforts.
Figure 5. Compounds 1b, 1c, and 1d showed a dose–response activation of the NRF2 reporter. HEK293T cells were cotransfected with the 3×ARE-LUC reporter and the Renilla control vector and treated with DMF (20 μM) as a positive control or three different concentrations of compounds 1 (6 μM in blue, 20 μM in green, and 60 μM in pink) for 16 h. Luciferase experiments were performed in triplicates at least twice. The values in graphs correspond to the mean ± S.E.M. To assess differences between groups, one-way ANOVA followed by a Tukey's multiple comparison post-test was performed. Asterisks denote statistically significant differences with ***p < 0.001 and ****p < 0.0001. The numerical data corresponding to this figure can be found in Table S3.

Table 7. Comparison of NRF2 Inducing Capacity of Compounds 1b–d, Expressed as CD (Concentration Needed to Double Luciferase Expression), and Their ROCK2 Inhibition Activity, Measured by IC50 Values, Showing that Compounds 1b and 1d Have a Well-Balanced Multitarget Profile

| compound | NRF2 induction, CD (μM) | ROCK2 inhibition, IC50 (μM) | NRF2/ROCK2 ratio |
|----------|------------------------|-----------------------------|------------------|
| 1b       | 12                     | 2.13                        | 5.2              |
| 1c       | 15                     | 0.32                        | 46.9             |
| 1d       | 1                      | 0.73                        | 1.4              |

To carry out the second approach, i.e., subcellular fractionation, we selected compound 1d, which had no adverse effects on cell viability and also induced the expression of 3×ARE-LUC in a dose-dependent manner, in a similar concentration range to DMF. We analyzed the subcellular distribution of NRF2 in SH-SY5Y cells after treatment with compound 1d at different time points. As shown in Figure 6, subcellular fractionation assays demonstrated that compound 1d induced a significant accumulation of NRF2 in the nucleus and, to a lesser extent, in the cytosol. These results showed that compound 1d could induce NRF2 transcriptional activation.

2.8. Compound 1d Activates the NRF2 Signature.

Next, we determined whether compound 1d could induce the activation of NRF2 target genes. Consistent with the results shown above, compound 1d increased the mRNA levels of NRF2-dependent genes including heme oxygenase 1 (HMOX1) and NAD(P)H dehydrogenase quinone 1 (NQO1) (Figure 7) in SH-SY5Y cells, in a time-dependent fashion. Moreover, this increase in mRNA levels resulted in an increase in the protein levels of HO-1 and NQO1, respectively, reaching a maximum after 24 h of treatment. The differences in the response times of HMOX1 and NQO1 are due to different activation kinetics, as described previously. All these results confirm that compound 1d effectively promotes the expression of the antioxidant response enzymes HO-1 and NQO1.

2.9. Compound 1d Induces the NRF2 Transcriptional Signature through KEAP1-Dependent Mechanisms.

As mentioned in the Introduction, there are several mechanisms by which the expression of NRF2 can be modulated by small organic molecules. The main control of NRF2 levels is due to its binding to the repressor KEAP1. This protein is a highly reactive redox sensor due to its 27 cysteine (Cys) residues and therefore could be a target of these small organic molecules.

We assessed the implication of KEAP1 on the activation of NRF2 by compound 1d by using mouse embryonic fibroblasts (MEFs) from wild-type (Keap1+/+) or KEAP1-deficient (Keap1−/−) mice. As shown in Figure 8, in Keap1+/+ cells, compound 1d increased the mRNA levels of HMOX1 and NQO1 in a dose-dependent manner, consistent with the results shown in Table 7.

Figure 6. Compound 1d-induced nuclear translocation of NRF2. SH-SY5Y cells were incubated in the presence of compound 1d (20 μM) for 1, 2, and 4 h, and subcellular fractions were analyzed by immunoblotting: upper panel, NRF2 levels; middle panel, GAPDH levels used as a cytosol protein loading control; lower panel, Lamin B level used as a nuclear protein loading control. Densitometric quantification of NRF2 protein levels of representative blots. Experiments were performed in duplicate at least twice. To assess differences between groups, one-way ANOVA followed by a Tukey's multiple comparison post-test was performed. Asterisks denote statistically significant differences with *p < 0.05, **p < 0.01, and ****p < 0.0001.
NQO1, in a time-dependent way. Again, we could observe the different kinetics of both genes. On the contrary, in Keap1−/− MEFs, compound 1d is not capable of inducing the expression of NQO1 and induced the expression of HMOX1 to a lesser extent than in the wild-type cells. The small increase observed in HMOX1 mRNA levels may be due to the activation of pathways independent of NRF2 since the HMOX1 promoter contains binding sites for other transcription factors. Our results confirm the regulation of NRF2 by compound 1d in a KEAP1-dependent manner. Although KEAP1-independent mechanisms cannot be completely ruled out, they are probably of less physiological significance.

Regarding the mechanistic details of KEAP1-dependent NFR2 induction, in most of the cases, it depends on Cys alkylation by electrophilic moieties. Alternatively, NRF2 inducers may act by directly interfering with the KEAP1–NRF2 protein–protein interaction. In order to further investigate the role of KEAP1 in the effect exerted by compound 1d, we assessed whether our compound could act by releasing NRF2 via inhibition of the protein–protein interaction. Fluorescence polarization and differential scanning fluorimetry assays indicated that compound 1d is not able to inhibit the NRF2–KEAP1 interaction at 100 μM or lower concentrations (Table 8). Thus, it can be concluded that 1d interacts with the sensor part of KEAP1. Unlike ferulic and cinnamic acids, 1d does not contain a side-chain α,β-unsaturated carbonyl moiety, and therefore, the reaction with a Cys residue cannot be due to a Michael addition onto such a...
functional group. On the other hand, the presence of the catechol structural fragment allows the oxidative generation of a highly electrophilic ortho-quinone species, which may bind covalently to the cysteine residues of KEAP1 that act as sensors. Other catechol structures such as dopaminochrome or oxidation products of entacapone, tolcapone, or apomorphine have been described, and they can be easily attacked by thiol groups, resulting in the release of NRF2 from KEAP1. Similarly, NRF2 induction by epigallocatechin-3-gallate has been explained via the prior oxidation of its catechol moieties to ortho-quinones.

2.10. Lymphoblasts from sALS and SOD1-ALS

Evidence Significant Differences at Basal Levels and after Induction of the NRF2 Signaling Pathway. Finally, once compound 1d-dependent induction of NRF2 was proven and characterized, we studied the effect of this compound in a human cell-based model recently developed in our group that mimics ALS. As previously described, we corroborated that in sALS lymphoblasts (without treatment), the NRF2 signature was significantly increased in comparison to the control or SOD1-ALS.
lymphoblasts (Figure 9), at the mRNA and protein levels. These data suggested important differences in the molecular mechanisms related to NRF2 signaling linked to the pathology between sALS and SOD1-ALS. Therefore, we analyzed the effect of the induction of the NRF2 signaling pathway by compound 1d (20 μM) in sALS and SOD1-ALS lymphoblasts, compared to controls. Treatment of control lymphoblasts with 1d did not produce changes in NEFL2 mRNA levels, as would be expected due to the fact that the NRF2 pathway is mainly regulated at the protein level. Indeed, the treatment with 1d significantly increased the levels of the NRF2 protein and consequently the levels of NRF2-dependent genes, HMOX1 and NQO1, both at the mRNA and protein levels (Figure 9). Lymphoblasts from sALS patients have high baseline NRF2 pathway activity compared to control lymphoblasts. Therefore, in lymphoblasts from patients with sALS, treatment with 1d did not produce a significant increase in NRF2 (compared to untreated sALS lymphoblasts) or the activity of its signaling pathway, either at the mRNA or protein levels. On the contrary, in the lymphoblasts from SOD1-ALS patients, where the basal levels of the NRF2 pathway were similar to the levels of the control cells, treatment with 1d is capable of inducing HMOX1 and NQO1 levels in a very significant way, both at the mRNA and protein levels. These results point to a personalized pharmacological strategy for patients with ALS, where modulation of NRF2 should be personalized, based on the molecular alterations displayed by the different types of patients. Our results underline the relevance of NRF2 activators for the treatment of SOD1-ALS patients.

3. CONCLUSIONS

A series of compounds were designed to combine the key structural fragments of the ROCK inhibitor fasudil and the NRF2 inducers/radical scavengers ferulic and caffeic acids and maintain the properties of the parent molecules, in a multitarget strategy. One of the compounds, a dihydrocaffeic acid hybrid 1d was selected for further studies due to its good profile and absence of cytotoxicity and was shown to induce the NRF2 signature by KEAP1-dependent mechanisms. In lymphoblasts obtained from SOD1-ALS patients, this compound significantly activated the NRF2 signature, while in sALS, it hardly produced induction, underscoring the potential of this compound in a personalized therapy of ALS, especially for the case of patients with SOD1 mutation, and showing the relevance of NRF2 activation as a therapeutic strategy for the treatment of SOD1-ALS patients. Thus, compound 1d can be viewed as an interesting hit on which to base future optimization efforts aimed at improving its activity and ADMET profile.

4. EXPERIMENTAL SECTION

4.1. General Experimental Information. All commercial reagents and solvents were used as received. Reactions were monitored by thin-layer chromatography on silica gel-coated aluminum plates containing a fluorescent indicator. Microwave-assisted reactions were performed using an CEM Discover focused microwave reactor. Separations by flash chromatography were performed on conventional silica gel columns or on a Combiflash Teledyne automated flash chromatograph. Melting points were measured with a Kofler-type microscope with a heating plate from Reichert, model 723, and were uncorrected. Infrared spectra were obtained with an Agilent Cary630 FTIR spectrophotometer with a diamond ATR accessory for solid and liquid samples, and wave-numbers are given in cm⁻¹. NMR data were obtained using a Bruker Avance spectrometer (CAI de Resonancia Magnética, UCM), working at 250 MHz for ¹H NMR and 63 MHz for ¹³C NMR; chemical shifts are given in parts per million (δ scale), and coupling constants (J) are given in Hertz. Combustion elemental analyses were obtained by the CAI de Microanalisi, Universidad Complutense, using a Leco CHNS-932 combustion microanalyzer. UV–vis measurements were taken with a UV–vis spectrophotometer Cary60 from Agilent, equipped with control and data acquisition Cary WinUV software. The purity of all compounds was >95%, as determined by combustion elemental analysis.

4.2. General Procedure for the Synthesis of Isoquinoline-5-sulfonamides 2. Isoquinoline-5-sulfonic acid (1 mmol), thionyl chloride (1 mL), and a catalytic amount of dimethylformamide (0.01 mmol) were refluxed at 70 °C under an argon atmosphere for 2 h. After this time, the cooled reaction mixture was filtered and washed twice with dichloromethane to afford isoquinoline-5-sulfonyl chloride as a white solid.

A solution of this chloride (1 mmol), pyridine (2 mmol), and triethylamine (1 mmol) in acetonitrile (5 mL) was added dropwise over 20 min to a solution of piperazine or homopiperazine (3 mmol) in acetonitrile (25 mL). During the addition, the temperature was maintained at −5 °C, and then, the reaction was left to warm to room temperature and stirred overnight. The reaction mixture was then concentrated under reduced pressure and redissolved in dichloromethane. The organic phase was then washed with water five times and concentrated under reduced pressure vacuum. The liquid afforded was then purified by chromatography in a silica gel (9:1 dichloromethane:methanol) to afford the pure compounds 2.

4.2.1. 5-(Piperazin-1-yl)sulfonil)isoquinoline (2a). Prepared from isoquinoline-5-sulfonic chloride (263 mg, 1 mmol), piperazine (258 mg, 3 mmol), pyridine (152 mg, 2 mmol), and triethylamine (0.14 mL, 1 mmol). Reaction time: 2 h. Yield: 180 mg (65%). Mp: 160–161 °C (lit., 162 °C). ¹H NMR (250 MHz, CDCl₃): δ 9.35 (d, J = 0.9 Hz, 1H), 8.68 (d, J = 6.2 Hz, 1H), 8.54 (dt, J = 6.2, 0.9 Hz, 1H), 8.37 (dd, J = 7.4, 1.3 Hz, 1H), 8.23 (d, J = 8.2 Hz, 1H), 7.72 (dd, J = 8.1, 7.5 Hz, 1H), 3.18–3.09 (m, 4H), 2.93–2.85 (m, 4H). ¹³CNMR (63 MHz, CDCl₃): δ 153.7, 145.5, 134.7, 134.3, 132.5, 129.5, 126.3, 113.0, 46.8, 45.8. 4.2.2. 5-(1,4-Diazepan-1-yl)sulfonil)isoquinoline 2b (Fasudil). Prepared from isoquinoline-5-sulfonic chloride (263 mg, 1 mmol), homopiperazine (300 mg, 3 mmol), pyridine (152 mg, 2 mmol), and triethylamine (0.14 mL, 1 mmol). Reaction time: 2 h. Yield: 247 mg (85%), as a viscous oil that solidifies upon standing for 2–3 days to yield a pale-yellow solid. Mp: 128–129 °C (lit., 130 °C). ¹H NMR (250 MHz, CDCl₃): δ 9.37 (d, J = 0.8 Hz, 1H), 8.72 (d, J = 6.2 Hz, 1H), 8.47 (dt, J = 6.2 Hz, 1H), 8.37 (dd, J = 7.4, 1.2 Hz, 1H), 8.22 (d, J = 8.2 Hz, 1H), 7.76–7.61 (m, 1H), 3.57–3.41 (m, 4H), 2.99 (dd, J = 11.7, 7.8, 4.7 Hz, 4H), 1.90 (s, 6H), 1.85 (dd, J = 12.0–6.0 Hz, 2H). ¹³CNMR (63 MHz, CDCl₃): δ 153.7, 145.5, 135.0, 133.8, 133.3, 132.0, 129.6, 126.3, 118.0, 51.4, 50.7, 48.0, 47.8, 31.4.

4.3. General Procedure for the Synthesis of Hybrid Compounds 1. The suitable starting material 2 (1 mmol) was dissolved in THF (10 mL) or a 1:1 mixture of dichloromethane and methanol (10 mL). The suitable cinnamic acid derivative (0.9 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (1 mmol), hydrated hydroxybenzotriazole (HOBt·H₂O) (1 mmol), and diisopropylethylamine or EtN₃ (2 mmol) were then added to the solution, which was stirred at room temperature until completion, as confirmed by TLC. The reaction mixture was then concentrated under reduced pressure and then redissolved in ethanol. The pure compounds (1) precipitated at reduced temperature (4 °C) or were purified by silica gel chromatography, using the conditions specified in each case.

4.3.1. 3-(3,4-Dihydroxyphenyl)-1-(4-(isooquinolin-5-sulfonil)-piperazin-1-yl)prop-2-en-1-one (1a). Prepared from 5-(1,4-piperazin-1-yl)sulfonil)isoquinoline (2a) (277 mg, 1 mmol), caffeic acid (162 mg, 0.9 mmol), EDCI (191 mg, 1 mmol), HOBT (153 mg, 1 mmol), and triethylamine (203 mg, 2 mmol). Reaction time: 72 h.
3.4. 3-(4-Hydroxy-3-methoxyphenyl)-1-(4-(isouquinolin-5-sulfonyl)isoquinolin-1-yl)sulfonyl)isoquinoline (2b) (291 mg, 1 mmol), ferulic acid (174 mg, 0.9 mmol), EDCI (191 mg, 1 mmol), HOBT (153 mg, 1 mmol), and triethylamine (203 mg, 2 mmol). Reaction time: 72 h. Yield: 70% (292 mg, 1 mmol).

The crystallized product was recrystallized from MeOH/MeOD (15:1) and dried under reduced pressure to obtain the AutoDock file. The AutoDock file was used to dock the ligand into the active site of the target protein. The docking scores were analyzed to identify the most suitable docked conformation of the ligand.
calculating the expected center of the interaction area \((x/y/z = 43.961/−8.203/103.693)\), and its size was of \(x/y/z = 22/26/20 \text{ Å}\). The exhaustiveness was 16, and the number of calculated conformation was 9. Processing of the ligands was performed with UCSF Chimer 1.1.4. The docking calculation was carried out by AutoDockVina.62

Molecular dynamics (MD) simulation was performed using Gromacs 2018.163 and CHARMM3664 was used as a force field. Topologies and parameters of both the ligand and the enzyme were created with CgenFF. The complex was solvated using an SPC water model and then minimized. A two-stage equilibration was performed by applying the NVT ensemble followed by the NPT ensemble for 50,000 ps of 2 fs each. A 10 ns simulation was calculated for each ligand and conformation, with a time step of 2 ps and a cutoff of 1.0 nm. The long-range electrostatic energies were calculated with the PME method, with a fourth-order cubic interpolation and a spaced grid of 0.16 nm. The temperature was regulated at 300 K using a Berendsen thermostat with a coupling constant of 0.1 ps. The pressure was fixed at 1 bar and controlled with a Parrinello–Rahman barostat with a coupling constant of 2 ps, and a compressibility of 4.5 × 10⁻⁵ bar⁻¹ was employed.

Root-mean-square displacement (RMSD) between any snapshot and the minimized state of the system was calculated to evaluate the equilibrium of the system during simulation. The mobility of the ligand around the enzyme was evaluated by measuring distances between atoms present in residues from the hinge region of the enzyme and different atoms present in the ligands. An estimation of the binding energy was also calculated by using the molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) method using the last 200 MD snapshots (2 ns) from each simulation.

### 4.5. Serum Albumin Binding

Human serum albumin (HAS) was diluted in phosphate buffer solution (\(pH = 7.2, 50 \text{ mM}, [\text{NaCl}] = 150 \text{ mM}\)) to a final concentration of 2 \(\mu\text{M}\). Then, compound 1b was sequentially added to achieve increasing concentrations (0.2, 0.8, 1.4, 2, 8, 14, and 20 \(\mu\text{M}\)). After each addition, the mixture was incubated at rt for 5 min, and then, the emission fluorescence spectra of HAS \((λ_\text{exc} = 280 \text{ nm})\) were measured using a Fluorometer Max-4P (Horiba Jobin Yvon). The data obtained were further processed using the Stern–Volmer and Scatchard models in GraphPad Prism 8.0 software.

### 4.6. Liver Microsome Stability Assay

Human liver microsomes and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Fisher Scientific SL. This assay provides information on the metabolic stability of early drug discovery compounds based on liver microsomes. Microsome stability was tested by incubating 8 \(\mu\text{M}\) of the test compound and verapamil (as control) with 1.0 mg/mL hepatic microsomes (pooled human liver microsomes) in 0.1 M potassium phosphate buffer (\(pH = 7.4\), with \(1 \times 10^{-5} \text{ M} \text{MgCl}_2\)). The reaction was initiated by adding NADPH (1 mM final concentration). Aliquots of 150 \(\mu\text{L}\) were collected at defined time points (0, 5, 15, 30, and 60 min) and added to cold acetonitrile (150 \(\mu\text{L}\) containing an internal standard (5 \(\mu\text{g/mL} \text{ warfarin}\)) to stop the reaction and precipitate the protein. After stopping the reaction, the samples were centrifuged at 4 °C for 15 min, and the loss of the parent compound was analyzed by high-pressure liquid chromatography coupled to mass spectrometry (HPLC-MS). Data were log transformed and represented as half-life. All experiments were conducted in duplicates.

### 4.7. Antioxidant Activity Test

The antioxidant activity was determined by the 2,2-diphenyl-1-pycrylhydrazyl hydrate (DPPH) assay.65 All the measurements were performed in an 80% MeOH solution, with the final reaction volume being 3 mL. A stock of DPPH solution in DMSO (10⁻² M) was prepared and stored at −20 °C, and the same procedure was performed with the antioxidant solution. Then, the DPPH stock solution was diluted to 2 mL with a final concentration of 150 \(\mu\text{M}\) (final volume of 3 mL and final concentration of 100 \(\mu\text{M}\)). The antioxidant stock was diluted to 1 mL at 3, 9, 30, 90, and 300 \(\mu\text{M}\) (final volume of 3 mL and final concentrations of 1, 3, 10, 30, and 100 \(\mu\text{M}\)). The mixture was incubated at room temperature for 30 min. Once this time had passed, the absorbance was measured in a spectrophotometer at 517 nm. Trolox, caffeine acid, and ferulic acid were used as a standard (1, 3, 10, 30, and 100 \(\mu\text{M}\) final concentrations). A blank using 80% MeOH solution instead of the corresponding antioxidant was used in each assay. Two independent measurements of each sample were performed. Data were processed using Origin software, and sigmoidal fitting according to the DoseResp function was performed to extrapolate the IC₅₀ (\(\mu\text{M}\)) of each antioxidant.

### 4.8. ROCK Inhibition Studies

ROCK-II (ROKa) (5–20 \(\mu\text{M}\) diluted in 50 mM Tris pH 7.5, 0.1 mM EGTA, 0.1% mercaptoethanol, and 1 mg/mL BSA) was assayed against the Long S6 substrate peptide (KEAKREQTEIAKRRRLSSL-RASTSKSGSSQK) in a final volume of 25.5 \(\mu\text{L}\) containing 50 mM Tris pH 7.5, 0.1 mM EGTA, 30 \(\mu\text{M}\) Long S6 substrate peptide, 10 mM magnesium acetate, and 0.02 mM [33P-g-ATP] (50–1000 cpm/pmol) and incubated for 30 min at room temperature. Assays were stopped by addition of 5 \(\mu\text{L}\) of 0.5 M (3%) orthophosphoric acid and then harvested onto P81 Unifilter plates with a wash buffer of 50 mM orthophosphoric acid.

### 4.9. Cell Culture and Reagents

Human embryonic kidney (HEK) 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 80 \(\mu\text{g/mL} \text{ gentamycin}\). Transient transfections were performed with calcium phosphate using reagents from Sigma. The Keap1⁻/⁻ mouse embryonic fibroblasts (MEFs) and their corresponding wild-type Keap1⁺/⁺ MEFs were kindly provided by Dr. Ken Itoh (Department of Stress Response Science, Center for Advanced Medical Science, Hirosaki University, Japan). MEFs were grown in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mm l-glutamine. SH-SY5Y cells were grown in DMEM and Ham’s F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The medium was changed to serum-free DMEM without antibiotics 16 h before treatments. DMF (cat. no. 242926, Sigma-Aldrich) was used at 20 \(\mu\text{M}\) for 16 h.

### 4.10. Lymphoblastic Cell Lines

Peripheral blood samples of all the individuals enrolled in this study were collected after written informed consent of the patients or their relatives (demographic information is presented in Table 9) to establish the lymphoblastoid cell lines (LCLs), by infecting peripheral blood lymphocytes with the Epstein–Barr virus (EBV), as previously described.66 Participants or their relatives gave written informed consent. This study was approved by the Hospital Doce de Octubre and the Spanish Council of Higher Research Institutional Review Boards. All patients were diagnosed by applying the revised El Escorial criteria.67 Control healthy individuals were recruited separately and did not have any

### Table 9. Demographic and Clinical Characterization of Subjects Included in This Study

| Gender (M/F) | Family history | Age range (M, F) | Site of onset (N) | Mutation (n) |
|--------------|----------------|-----------------|-----------------|-------------|
| control (n = 7) | SALS (n = 6) | SOD1-ALS (n = 4) |
| S/2 | 3/3 | 4/0 |
| no | no | yes |
| at sampling | 52–75 | 55–76 | 46–54 |
| bulbar | NA | 4 |
| spinal | NA | 1 | 4 |
| respiratory | NA | 1 |
| SOD1 het N65S | 1 |
| SOD1 | 2 |
| SOD1 het p.Leu117Val | 1 |
| SOD1 het p.Asn139His | 1 |

*M, male; F, female; NA, not applicable.*
known neurological disorder. Genetic testing for SOD1, TARDBP, FUS, and C9ORF72 was performed in all cases. LCLs were grown in suspensions in T flasks, in an RPMI-1640 medium containing 2 mM l-glutamine, 100 μg/mL streptomycin/penicillin, and 10% (v/v) fetal bovine serum (FBS) and maintained in a humidified 5% CO₂ incubator at 37 °C.

4.11. MTT Viability Assay. HEK293T cells were plated in 24-well culture plates (75,000 cells/well) and incubated in a CO₂ incubator. The next day, treatment was given according to the experimental requirement. Sixteen hours later, 50 μL of MTT solutions from the stock (5 mg/mL) was added, and cells were incubated in a CO₂ incubator in the dark for 2 h. The medium was removed, and formazan crystals formed by the cells were dissolved using 500 μL of DMSO followed by transfer in 96-well plates. The absorbance was read at a 570 nm wavelength on a multwell plate reader.70

4.12. Plasmids and Luciferase Assay. Transient transfections of HEK293T cells were performed with the expression vectors for TK-Renilla (Promega, Madison, CA) and the expression vector ARE-LUC (Dr. J. Alam, Dept. of Molecular Genetics, Ochsner Clinic Foundation, New Orleans, LA). Cells were seeded on 24-well plates (75,000 cells per well), cultured for 16 h, and transfected using calcium phosphate. Eight hours after transfections, cells were treated with 3 different concentrations of the compounds (6, 20, and 60 μM). DMF (20 μM) was used as a positive control. After 16 h, the cells were lysed and assayed with a dual-luciferase assay system (Promega) according to the manufacturer’s instructions. Relative light units were measured in a GloMax 96 microplate luminometer with dual injectors (Promega).

4.13. Preparation of Nuclear and Cytosolic Extracts. SH-SY5Y cells were seeded in p100 plates (1 × 10⁶ cells/plate) and treated with 20 μM compound 1d. Cytosolic and nuclear fractions were prepared as described previously.71 Brieflly, cells were washed with cold PBS and harvested by centrifugation at 1100 rpm for 10 min. The cell pellet was resuspended in 3 pellet volumes of cold buffer A (20 mm HEPES, pH 7.0, 0.15 mm EDTA, 0.015 mm EGTA, 10 mm KCl, 1% Nonidet P-40, 1 mm phenylmethylsulfonyl fluoride, 20 mm NaF, 1 mm sodium pyrophosphate, 1 mm sodium orthovanadate, and 1 μg/mL leupeptin) and incubated in ice for 30 min. Then, the homogenate was centrifuged at 500g for 5 min. The supernatants were taken as the cytosolic fraction. The nuclear pellet was resuspended in 5 volumes of cold buffer B (10 mm HEPES, pH 8.0, 0.1 mm EDTA, 0.1 mm NaCl, 25% glycerol, 1 mm phenylmethylsulfonyl fluoride, 20 mm NaF, 1 mm sodium pyrophosphate, 1 mm sodium orthovanadate, and 1 μg/mL leupeptin). After centrifugation in the same conditions indicated above, the nuclei were resuspended in loading buffer containing 0.5% SDS. The cytosolic and nuclear fractions were resolved in SDS-PAGE and immunoblotted with the antibodies indicated in Supporting Information Table S4.

4.14. Immunoblotting. Whole cell lysates were prepared in RIPA-buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Igepal, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin). Whole cell lysates, cytosolic and nuclear fractions containing 25 μg of whole proteins from SH-SY5Y, or lymphoblast-treated cells were loaded for SDS-PAGE electrophoresis. Immunoblots were analyzed as described previously.72 The primary antibodies used are described in Supporting Information Table S4.

4.15. Analysis of mRNA Levels by Quantitative Real-Time PCR. Total RNA extraction, reverse transcription, and quantitative polymerase chain reaction (PCR) were done as detailed in previous articles.72 Primer sequences are shown in Supporting Information Table S5. Data analysis was based on the ΔΔCT method with normalization of the raw data to housekeeping genes (Applied Biosystems). All PCRs were performed in triplicates.

4.16. Statistical Analyses. Data are presented as means ± SEM. To determine the statistical test to be used, we employed GraphPad Instat 3.1, which includes the analysis of the data to normal distribution via the Kolmogorov–Smirnov test. In addition, statistical assessments of differences between groups were analyzed (GraphPad Prism 6, San Diego, CA) by unpaired Student’s t-tests when normal distribution and equal variances were fulfilled or by the nonparametric Mann–Whitney test. One- and two-way ANOVA with the post hoc Newman–Keuls test or Bonferroni’s test was used, as appropriate.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c01255.

Complementary experimental data and copies of spectra (PDF)
PDB files for fasudil and compound 1d docked in ROCK2 (PDB)
Molecular formula strings (CSV)

AUTHOR INFORMATION

Corresponding Authors

Israel Lastres-Becker — Instituto de Investigaciones Biomédicas “Alberto Sols” UAM-CSIC, Department of Biochemistry, School of Medicine, and Institute Teofil Hernando for Drug Discovery, Universidad Autónoma de Madrid, 28029 Madrid, Spain; Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, 28031 Madrid, Spain; Phone: +34915854449; Email: ilbecker@ibi.unam.es

J. Carlos Menéndez — Unidad de Química Orgánica y Farmacéutica, Departamento de Química en Ciencias Farmacéuticas, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain; orcid.org/0000-0002-0560-8416; Phone: +3439418440; Email: josecm@farm.ucm.es

Authors

Olmo Martín-Cámara — Unidad de Química Orgánica y Farmacéutica, Departamento de Química en Ciencias Farmacéuticas, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain

Marina Arribas — Instituto de Investigaciones Biomédicas “Alberto Sols” UAM-CSIC, Department of Biochemistry, School of Medicine, and Institute Teofilo Hernando for Drug Discovery, Universidad Autónoma de Madrid, 28029 Madrid, Spain

Geoffrey Wells — UCL School of Pharmacy, University College London, London WC1N 1AX, United Kingdom; orcid.org/0000-0002-0253-911X

Marcos Morales-Tenorio — Centro de Investigaciones Biológicas Margarita Salas, CSIC, 28040 Madrid, Spain

Ángeles Martín-Requero — Centro de Investigaciones Biológicas Margarita Salas, CSIC, 28040 Madrid, Spain; Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, 28031 Madrid, Spain

Gracia Porras — Centro de Investigaciones Biológicas Margarita Salas, CSIC, 28040 Madrid, Spain

Ana Martínez — Centro de Investigaciones Biológicas Margarita Salas, CSIC, 28040 Madrid, Spain; Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, 28031 Madrid, Spain; orcid.org/0000-0002-2707-8110

Giorgio Giorgi — Unidad de Química Orgánica y Farmacéutica, Departamento de Química en Ciencias Farmacéuticas, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain
Author Contributions
OM-C. and MA. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. JC.M., AM., PL-A., and IL-B. performed conceptualization; MA., OM-C., GP., GW., MM-T., and IL-B. performed the methodologies; OM-C., MA., GP., AM-R., PL-A., JC.M., and IL-B. performed formal analysis; JC.M., PL-A., AM-R., AM., and IL-B. acquired resources; JC.M., AM-R., AM., PL-A., and IL-B. wrote the original draft; JC.M. and IL-B. reviewed and edited the manuscript; all authors performed visualization; JC.M., AM-R., AM., GG., PL-A., and IL-B. supervised the study; JC.M., PL-A., AM-R., AM, and IL-B. acquired funding. All authors have read and agreed to the published version of the manuscript.

Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
ADME, absorption, distribution, metabolism, and excretion; AKT, protein kinase 1; ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; ARE, antioxidant response element; C9orf72, chromosome 9 open reading frame 72; CUL3/RBX1, cullin 3 and RING-box protein1; DMF, dimethyl fumarate; DPPH, 2,2-diphenyl-1-picrylhydrazyl hydrate; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ERK, extracellular signal-regulated kinase; fALS, familial amyotrophic lateral sclerosis; FDA, food and drug administration; FUS, fused in sarcoma (protein); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK-3, glycogen synthase kinase 3; HMOX1, heme oxygenase 1; HOβt, 1-hydroxybenzotriazole; JNK, c-Jun N-terminal kinase; KEAP1, cap’n’collar homology (ECH)-associated protein 1; LUC, luciferase; MAPK, mitogen-activated protein kinase; MF2s, mouse embryonic fibroblasts; MM-PBSA, molecular mechanics Poisson–Boltzmann surface area; MNs, motoneurons; MTDL, multitarget-directed ligand; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NQO1, NAD(P)H dehydrogenase quinone 1; NRF2, nuclear factor erythroid 2-related factor 2; PAINS, pan-assay interference compounds; ROCK, Rho-associated protein kinase; sALS, sporadic amyotrophic lateral sclerosis; SOD1, superoxide dismutase 1; TDP-43, TAR DNA-binding protein 43; TLC, thin-layer chromatography; β-TrCP, β-transducin repeat-containing protein.

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