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Neuroprotective effects of a new self-immolative prodrug against copper-induced toxicity

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Abstract

Alzheimer's disease (AD) is an age related neurodegenerative disease. Protein misfolding and aggregation are considered to be the main pathological hallmarks. However, recent accumulating evidence suggest that metal dyshomeostasis and oxidative stress-induced damage are both involved in AD physiopathology. Copper is an essential trace element and, in addition to many essential biological functions, it is a redox active metal that generates deleterious reactive oxygen species. In turn, reactive oxygen species are responsible for macromolecular damage and neuronal cell death. Due to these findings, copper chelation seems to be a promising therapeutic target in Alzheimer's disease. RC-proof-ALZ is a multi-target compound whose function is to complex copper, lowering copper levels and acting as a trigger for a cascade of chemical reactions yielding to the release of H_2S. In this study we demonstrate that RC-proof-ALZ improves cell survival and protects N2a neuroblastoma cells from copper-induced cell toxicity by activating MAPK/ERK pathway and also that the activation of NF-κB pathway and the inhibition of a caspase-dependent apoptotic pathway might be involved in neuroprotection.

Introduction

Alzheimer's disease is a neurodegenerative disease and the most common cause of dementia in the elderly. There are four consistent features in the AD brains: (1) extracellular deposition of amyloid-β (Aβ) peptide, the major component of senile plaques; (2) intracellular hyperphosphorylation of the microtubules associated protein Tau; (3) elevated oxidative stress to lipids, proteins and nucleic acids and (4) a loss of biometal homeostasis. [1]

The role of metals in the physiopathology of Alzheimer's Disease (AD) has gained considerable relevance in recent years, with both *in vitro* and *in vivo* data demonstrating that a mismetabolism of metal ions may affect various cellular cascades ultimately leading to the development and/or potentiation of AD.[2] Copper, zinc and iron are three essential trace elements responsible for the function of many cellular enzymes and proteins, [3] and their distribution in the body, metabolism and excretion needs to be proper regulated. [4] Altered levels of neuronal metal ions in AD-affected areas including accumulation in protein deposits, and the interplay between metal ions and AD pathological proteins indicates a close relationship between protein misfolding, aggregation, and metal ion homeostasis. [5]

Copper is a highly reactive transition metal which is essential for electron-transfer reactions in biological systems. It is an integral part of many intracellular and extracellular copper-dependent enzymes and structural proteins and it is involved in numerous biochemical and physiological functions. [6]. For a number of enzymes, Cu(II) acts as biological activity center, such as Cu,Zn-superoxide dismutase (Cu,Zn-SOD), which catalyzes the dismutation of superoxide into H_2O_2 and O_2, ceruloplasmin, a protein involved in iron metabolism through its ferroxidase activity, cytochrome c oxidase, tyrosinase and dopamine β-hydroxilase.[1]

In addition to these essential structural and catalytic roles in diverse biological processes and due to ability to facilitate redox reactions and alternate between oxidized (cupric Cu^{2+}) and reduced (cuprous Cu^{+}) state when present in unbound form, copper ions can generate deleterious reactive oxygen species (ROS). [6] ROS is a term for a category of active oxygen-containing compounds generated from aerobic metabolism. [7] They are mainly produced by the Fenton and Haber-Weiss reaction. When transient metals such as copper (Cu^{+}) react with hydrogen peroxide (H_2O_2), oxidized copper, hydroxyl radical (·OH) and hydroxyl anion ($^{-}$OH) are produced. Then hydroxyl ion (·OH) reacts with H_2O_2 again and superoxide ($O_2^{\cdot-}$) is produced. [8] The superoxide radical is considered as the "primary" ROS, capable of further interactions with other molecules to generate other ROS [9] such as H_2O_2 and hydroxyl radicals. The latter are highly unstable radicals which could damage cellular component and will ultimately lead to cell death. [9]

When the levels of ROS overwhelm the intracellular antioxidant defense mechanism, a condition termed oxidative stress results [6] and macromolecular damage occurs. Modifications in DNA, RNA, membrane proteins, and phospholipids by free radicals impose deleterious consequences to cellular functions and can potentially initiate cascades of molecular aberration within the cell. [10] A growing number of evidence suggests that copper-catalyzed oxidative stress and oxidation of proteins (by ROS themselves and by end-products of lipid peroxidation such as 4-hydroxynonenal) are involved in AD. [6] AD is characterized by elevations in oxidatively damaged RNA, DNA, proteins, and phospholipids, and the damages temporally precede the appearance of hallmark AD pathologies, such as neurofibrillary tangles (NFT) deposition and Aβ aggregation. [10]

Besides the production of ROS, a role for copper, as well as for other metals, in neurodegeneration emerges from the direct binding at specific sites in the involved proteins.[3] In AD brains Cu(II) ions binds to amyloid-β peptides with high affinity and subsequently incurs the secondary structure changes and deposition of Aβ, the major component of senile plaques in AD brains.[1][11] In fact the aggregation of Aβ is promoted by copper and its neurotoxicity depends on catalytically generated H_2O_2 by Aβ-copper complexes *in vitro*. [3] Due to these findings, selective copper chelating is a promising therapeutic strategy. Copper sequestration would ultimately help preventing the generation and accumulation of ROS that incur oxidative damage to neuronal tissue and would simultaneously prevent the aggregation and deposition of Aβ-peptide. [10] The first generation of ligands were capable of removing Cu(II) from Aβ-peptides, stopping ROS production and favoring the disaggregation of Aβ senile plaques. Clioquinol and PBT2 have shown to be the best candidates and have gone under clinical trials, however both have failed in phase II. Different approaches are under focus to find new generations of ligands: (1) prochelators, which can be activated by H_2O_2 or β-secretase directly in brain; (2) multi-target compounds and (3) Cu(II)-chelators with a glucose moiety or nanoparticles as transporters through the blood brain barrier (BBB). [12]

In this work have we tested RC-proof-ALZ, a self-immolative prodrug. Self-immolative chemistry is based on the cascade of disassembling reactions triggered by the adequate stimulation leading to a sequential release of the smaller constituent elements. First the trisubstituted pyridine complexes with copper and thiocarbamate is released. This thiocarbamate is converted into carbonyl sulfide (COS) and allylamine. Later, carbonyl sulfide (COS) is hydrolyzed to hydrogen sulfide (H_2S) and carbon dioxide (CO_2) by the ubiquitous enzyme carbonic anhydrase (CA). This is termed Smart Multitarget Approach i.e. the pyridine acts as trigger for a cascade of reactions when in complex with copper and as copper chelator lowering the levels of free copper. As mentioned above, hydrogen sulfide (H_2S) is produced. Increasing evidence from both *in vivo* and *in vitro* studies suggest that H_2S has potential therapeutic value for treatment in AD. [14] Besides, H_2S shows potent anti-inflammation effects in animal models and exhibits antioxidant properties and protective effects against reactive oxygen species (ROS). [15]

Objectives

The aim of this work is to study the neuroprotective capacity of RC-proof-ALZ in N2a neuroblastoma cells after copper-induced cell toxicity by analyzing cell survival and activation or inhibition of several signaling pathways.

Results

We have carried out an *in vitro* study evaluating the neuroprotective role of RC-proof-ALZ in N2a neuroblastoma cells. First, cells were cultured reaching a cell confluency of 80%. Cell toxicity was induced by exposure to $CuSO_4$ 200 μM or H_2O_2 400 μM and cell survival was assessed after 24 hours treatment with RC-proof-ALZ at concentrations ranging from 100 nM to 20 μM. Second, we have analyzed different signaling pathways by Western blot.

RC-proof-ALZ protects N2a cells against Copper toxicity, but not H_2O_2

In order to study how RC-proof-ALZ affect cell survival in N2a neuroblastoma cells after 24 hours exposure to $CuSO_2$ 200 μM or H_2O_2 400 μM, cells were fixed and stained with DAPI to visualize nuclei. Nuclei were examined by analyzing fluorescence microscopy images. Cell density was assessed by counting nuclei in a known area and represented as percentage (**Figure 1**). After 24 hours treatment with $CuSO_4$ 200 μM + RC-proof-ALZ we have observed an increase of 22,26% in cell survival at RC-proof-ALZ 1 μM compared to $CuSO_4$ 200 μM treatment alone. By contrast, when toxicity was induced by H_2O_2 any of the tested concentrations of RC-prof-ALZ (100 nM to 20 μM) have revealed ameliorations in cell survival.

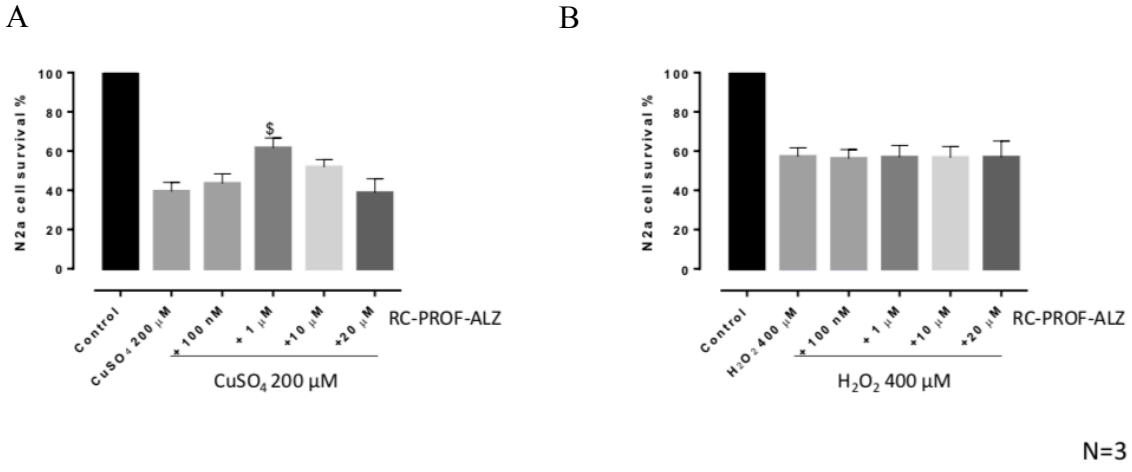

Figure 1. (A) Summary graph of cell survival after 24 h exposure to RC-proof-ALZ + $CuSO_4$ 200 μM in N2a neuroblastoma cells. At RC-proof-ALZ 1 μM survival levels increase 22,26% compared to $CuSO_4$ control ($p<0,005 vs CuSO4 200 μM). **(B)** Summary graph of cell survival after 24 h exposure to RC-proof-ALZ + H_2O_2 400 μM. No cell survival is observed compared to H_2O_2 control.

RC-proof-ALZ does not affect AKT signaling pathway in N2a neuroblastoma cells

Akt is one of the main downstream target of PI3K pathway and a pivotal kinase in neuronal survival. The active form of Akt has shown to be involved in neuronal viability maintenance by regulating the actions of several downstream effectors. [18] After 24 hours exposure of N2a neuroblastoma cells to $CuSO_4$ 200 μM + RC-proof-ALZ we did not observe any changes in Akt phosphorylation (**Figure 2**).

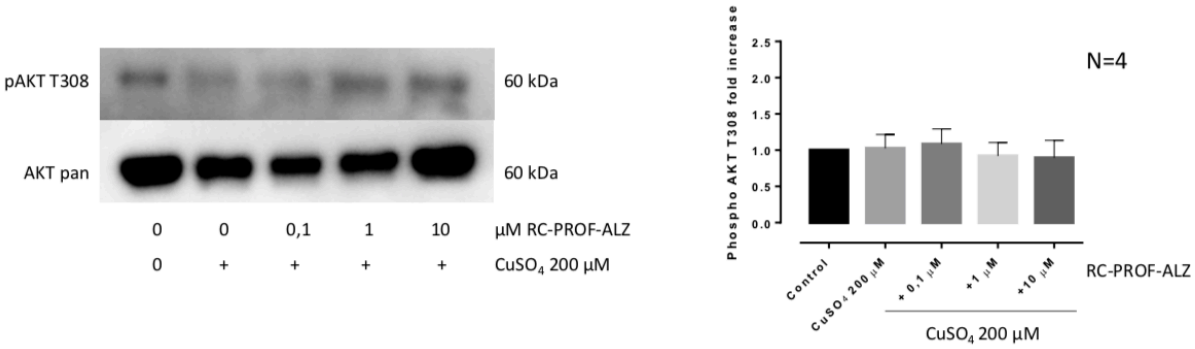

Figure 2. Phospho Akt levels after 24 hours exposure to RC-proof-ALZ + $CuSO_4$ 200 μM in N2a neuroblastoma cells. Representative western blot showing pAKT levels at increasing concentrations of RC-proof-ALZ and summary graph of pAkt T308 levels. No significant changes are observed to any RC-proof-ALZ concentrations (0,1 μM to 20 μM).

RC-proof-ALZ activates pERK signaling pathway

ERK1 (44 kDa) and ERK 2 (42 kDa) are closely related protein kinases of the MAP kinase family. ERK1/2 is regulated by a cascade of phosphorylations and activated by dual phosphorylation at Thr/Tyr residues of the ERK1/2 activation domain. This pathway affects multiple targets that may mediate its pro-survival activity. These include transcription factors that could stimulate production of anti-apoptotic mediators. [19] MAPK signaling pathway was assessed by Western blot, using an antibody that specifically recognizes the dually phosphorylated (active) form ERK1/2 (pERK). After 24 hours exposure of N2a neuroblastoma cells to $CuSO_4$ 200μM + RC-proof-ALZ, ERK1/2 phosphorylation levels raised 14,74 ± 2,95 times. This was significantly higher compared to $CuSO_4$ 200 μM treatment alone (**Figure 3**). Since the activation of ERK pathway is involved in anti-apoptotic processes and cell survival, [19] this result seems to be indicative for neuroprotection. Changes in ERK signaling pathway were also tested in N2a neuroblastoma cells after exposure to H_2O_2 400 μM + RC-proof-ALZ for 24 hours by measuring pERK levels. Here, we observed a 2,82 ± 0,81-fold increase at RC-proof-ALZ 1 μM. Control experiment in cells treated for 24 hours only with RC-proof-ALZ revealed no significant alterations in ERK phosphorylation.

In order to replicate our results in a more physiological model we have also carried out an *in vitro* study by using primary hippocampal cultured rat neurons. After 12 DIV we exposed them to a 24 hours treatment with $CuSO_4$ 200 μM + RC-proof-ALZ observing a 17,96 ± 0,81-fold increase in phosphorylated ERK. (**Figure 4**).

RC-proof-ALZ activates NF-κB signaling pathway in N2a neuroblastoma cells

Nuclear factor kappa B (NF-κB) family of transcription factor is responsible for the regulation of numerous multiple target genes. NF-κB is known for its role in preventing apoptotic cell death [16]. Five different mammalian NF-κB family members have been identified: NF-κB1 (also known as p105), NF-κB2 (also known as p100), RelA, RelB and c-Rel. [22] The members of the NF-κB family bind themselves forming homo- and heterodimers that are present in the inactive forms [16] complexed with IκB-a. Phosphorylation of IκB-a results in activation of NF-κB and translocation of the active NF-κB dimmers to the nucleus activates specific target genes. [23] In order to study an eventual role on the NF-κB pathway in the neuroprotective effect lead by RC-proof-ALZ in N2a neuroblastoma cells, we used p50 as a readout of the NF-κB pathway activation. After 24 hours exposure to RC-proof-ALZ + $CuSO_4$ 200 μM or H_2O_2 400 μM, the increase in p50 expression was not significant. However, the control experiment showed an increase in p50 levels after RC-proof-ALZ treatment alone in a dose dependent manner. p50 levels raised 2,082 ± 0,305 times at RC-proof-ALZ 1 μM (**Figure 3**).

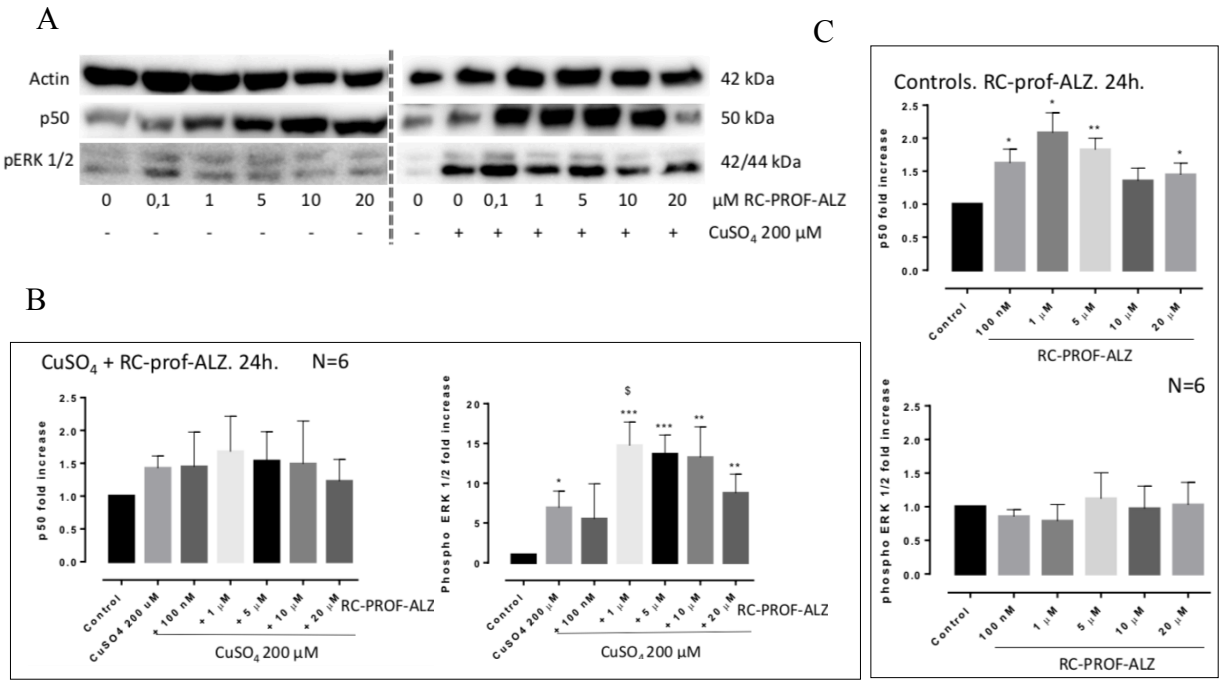

Figure 3. Activation of MAPK and NF-κB pathways after 24 hours exposure to indicated treatments in N2a neuroblastoma cells. **(A)** Representative western blot showing p50 y pERK1/2 levels. **(B)** 24 hours exposure to $CuSO_4$ 200 μM + RC-proof-ALZ. pERK1/2 levels increase significantly at RC-proof-ALZ concentrations ranging from 1 μM to 20 μM compared to controls. Compared to $CuSO_4$ control, only RC-proof-ALZ 1 μM increases pERK1/2. At the same conditions, no statistically significant increase in p50 levels is observed. **(C)** Summary graphs of control experiments performed in same conditions without $CuSO_4$. p50 levels increase 2,082 ± 0,305 times at RC-proof-ALZ 1 μM. No changes in pERK1/2 levels are observed. (*p<0,05, **p<0,001, ***p<0,0001 vs control). ($p<0,05 vs $CuSO_4$ 200 μM).

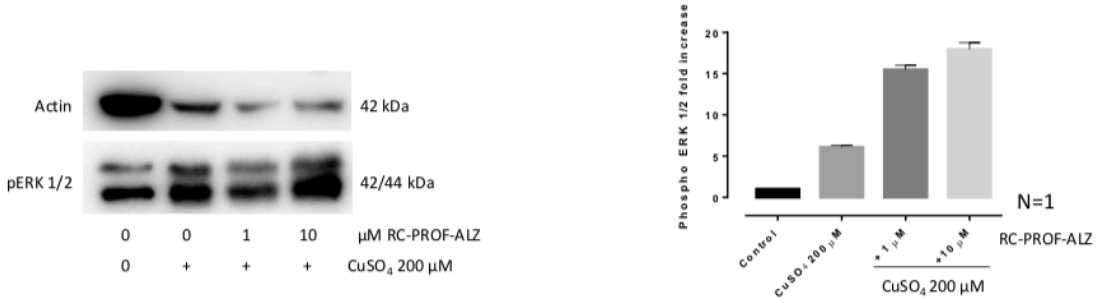

Figure 4. Representative western blot showing pERK1/2 levels at 12 DIV primary cultured rat hippocampal neurons exposed for 24 hours to $CuSO_4$ 200 µM + RC-proof-ALZ. Summary graph represents ERK1/2 phosphorylation levels which are increased 17,96 ± 0,81 times at RC-proof-ALZ 10 µM. No statistics were performed since it represents only one experiment.

RC-proof-ALZ could inhibit apoptosis after 24 hours treatment with 200 µM CuSO4

Caspases activation is considered specific to the apoptotic process and defines an irreversible stage in the cell death process. [26] Initiator caspases are strategically located in the apoptotic cascade between apoptotic stimulus receipt and effector caspase activation. Thus, they serve to integrate pro-death stimuli and transduce the death signal to downstream killer molecules. [24] The execution of apoptosis is controlled at least in part, by Bcl-2 family members that localize to the outer mitochondrial membrane and control mitochondrial permeability. [20] Pro-apoptotic Bax induces permeation of the outer mitochondrial membrane and elicits a pro-apoptotic response by stimulating the release of cytochrome c, which is blocked by Bcl-2. Cytochrome c, when released from the mitochondria to the cytoplasm, participates in the formation of a complex, known as the "apoptosome". This yields to the activation of caspase-9, which sets in motion the activation of a cascade of effectors, such as caspase-3, that induces cells death by irreversible proteolysis of critical cellular constituents. [20] Exposure to excess copper has shown to decrease transcriptional expression of pro-survival Bcl-2 gene, while the expression of typical pro-apoptotic Bax gene is increased in a concentration-dependent manner. [6] Activation of caspase-3 is a hallmark of apoptotic cell death and precedes changes in nuclear morphology. [17] We assessed caspase-3 activity by Western blot using the active form of the protease (cleaved caspase-3). RC-proof-ALZ at concentrations ranging from 1 µM to 20 µM has shown to markedly decrease caspase-3 activity. However, we have only carried out one experiment, thus this result is statistically not significant.

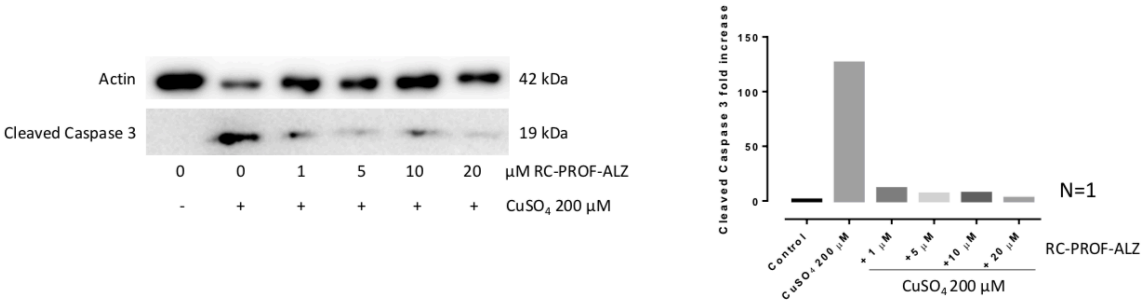

Figura 5. Measurement of cleaved caspase 3 in N2a neuroblastoma cells after 24 hours exposure to $CuSO_4$ 200 µM + RC-proof-ALZ. Representative western blot showing cleaved caspase-3 levels after RC-proof-ALZ treatment and summary graph of cleaved caspase 3 levels. Cleaved caspase-3 levels are 126,30-fold higher in $CuSO_4$ 200 µM controls. After addition of RC-proof-ALZ to $CuSO_4$ treatment at concentrations ranging from 1 to 20 µM, cleaved Caspase 3 levels show a 2,40-fold increase compared to control. No statistics were performed since it represents only one experiment.

Discussion

Our experiments have revealed an improvement in cell survival after RC-proof-ALZ treatment, demonstrating that RC-proof-ALZ protects N2a neuroblastoma cells from copper toxicity. Indeed, this improvement was only observed in copper-induced cell toxicity but not in cells exposed to H_2O_2 toxicity factor. These findings are consistent with the proposed mechanism of action of RC-proof-ALZ. As described previously, RC-proof-ALZ complexes with copper, depleting the levels of free copper as well as acting as trigger for further chemical reactions, which result in the release of H_2S. This might suggest that both, the decrease in copper levels and the increase in H_2S levels are responsible for the neuroprotective effect of RC-proof-ALZ.

Neurons respond to cell damage by activation of death signaling pathways. However, at the same time, cells may also mobilize defense mechanisms in an attempt to counteract cell death and enable damage repair. [19] Our results have shown that the neuroprotection by RC-proof-ALZ is mediated by activation of MAPK/ERK1/2 pathway and suggest that NF-κB pathway might be involved. PI3K-Akt pathway is also particularly relevant for mediating neuronal survival. [20] However, our data indicate that Akt signaling pathway is not involved in neuroprotection by RC-proof-ALZ in copper-induced cell toxicity.

The extracellular signal regulated kinase 1 (44 kDa) and 2 (42 kDa) (ERK 1/2) are members of the mitogen activated protein kinase (MAPK) family [21]. The MAPKs, including ERK1/2 are involved in the survival, proliferation and differentiation of nervous cells. Previous studies have shown that $CuSO_4$ itself increases ERK phosphorylation levels. [25] In our conditions we observed that $CuSO_4$ 200 μM + RC-proof-ALZ 1 μM significantly increased ERK phosphorylation compared to both control conditions and $CuSO_4$ treatment. This result suggest that MAPK/ERK1/2 pathway is involved in neuroprotection. The finding that RC-proof-ALZ increases ERK phosphorylation only in presence of $CuSO_4$ but not in control experiments in absence of copper supports the theory that RC-proof-ALZ is activated when complexed with copper.

We have carried out an experiment performed on primary cultured hippocampal rat neurons in same experimental conditions, observing an increase in ERK phosphorylation levels. However, we need more experiments to confirm these data. Besides, in these primary cultures, hippocampal neurons are co-cultured with glial cells (astrocytes) and it would be interesting to tackle if there are changes in the neuroprotective action of RC-proof-ALZ over different cell types (neurons and glial cells). More experiments need to be performed to answer this issue.

Nuclear factor kappa B (NF-κB) family of transcription factors is responsible for the regulation of numerous multiple-target genes involved in the inflammatory, immune reactions, cell proliferations, apoptosis or central nervous system (CNS) functioning. [16] Interestingly, NF-κB signaling pathway is one of the major neuroprotective pathways identified to be protective against Alzheimer's disease. A reduction of this protective NF-κB activation within Alzheimer's patients' brains might be one of the reasons for increased neurodegeneration. [23] We observed a raising trend in p50 levels after addition of $CuSO_4$ 200 μM + RC-proof-ALZ. These particular experiments showed a high variability thus these results are not significant and further experiments need to be done. Positive results might indicate that RC-proof-ALZ activates NF-κB pathway and could be involved in neuroprotection. In addition, p50 activation was only observed in control experiments after 24 hours exposure to RC-proof-ALZ in absence of copper. This is not concordant with the theory that RC-proof-ALZ is a prodrug activated in presence of copper and indicates that RC-proof-ALZ before cleaving might be involved in NF-κB activation. Further investigation of this pathway is needed to characterize its implication in RC-proof-ALZ mediated neuroprotection.

Caspases are critically involved in apoptotic death. They can serve both as initial transducers of apoptotic stimuli and final executioners of death. Several articles have reported caspase activation in either AD brain or AD-related experimental models. Caspase-9 plays and important role in mammalian nervous system development and is the dominant initiator caspase in the intrinsic apoptotic pathway. Caspase-9 activation triggers caspase-3 cleavage in most neuronal cells. [24] This pathway has shown to be the executor of neuronal apoptosis in N2a neuroblastoma cells after exposure to copper. Our preliminary data suggest that RC-proof-ALZ might protect neurons via inhibition of caspase-3 pathway. Even if the decrease in cleaved-caspase-3 activity after exposure to RC-proof-ALZ is evident, these data are statistically not significant. Only one experiment was performed because cleaved-caspase-3 antibody didn't work as desired. However, the result motivates to continue studying this pathway in further experiments.

Conclusions

1. RC-proof-ALZ improves cell survival in N2a neuroblastoma cells after copper-induced toxicity but not after exposure to H_2O_2. This indicates that copper is necessary for prodrug activation.
2. Concerning signaling pathways, neuroprotection by RC-proof-ALZ against copper-induced toxicity in N2a neuroblastoma cells is mediated by activating MAPK/ERK pathway.
3. PI3K-Akt signaling pathway is not involved in neuroprotection by RC-proof-ALZ in copper induced cell toxicity.
4. Our data suggest that NF-κB pathway is not involved in RC-proof-ALZ mediated neuroprotection against copper toxicity. However, the variability of the results and the non-significant raising trend in p50 expression prompted us to perform a new set of experiments in order to confirm these initial findings.

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