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Review article

The emerging role of mixed lineage kinase 3 (MLK3) and its potential as a target for neurodegenerative diseases therapies

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ABSTRACT

Selective and brain-permeable protein kinase inhibitors are in preclinical development for treating neurodegenerative diseases. Among them, MLK3 inhibitors, with a potent neuroprotective biological action have emerged as valuable agents for the treatment of pathologies such as Alzheimer's, Parkinson's disease and amyotrophic lateral sclerosis. In fact, one MLK3 inhibitor, CEP-1347, reached clinical trials for Parkinson's disease. Additionally, another compound called prostetin/12k, a potent and rather selective MLK3 inhibitor has started clinical development for ALS based on its motor neuron protection in both *in vitro* and *in vivo* models.

In this review, we will focus on the role of MLK3 in neuron-related cell death processes, neurodegenerative diseases, and the potential advantages of targeting this kinase through pharmacological modulation for neuroprotective treatment.

1. Introduction

Protein kinases play crucial roles in several biological processes. These enzymes regulate the activity of other proteins through a process known as phosphorylation. This is a post-translational modification (PTM) in which the gamma phosphate of ATP is catalytically transferred to a threonine, tyrosine, or serine of a substrate target [1]. This PTM is responsible for protein activation/deactivation, protein stability, protein interactions, and subcellular localization [2].

There are 518 protein kinases within the human kinome. Since the discovery of the first kinase inhibitor, Imatinib, pharmaceutical industries have increased their efforts to develop new protein kinase drugs. Today, there are 103 kinase inhibitors on the market, 82 FDA-approved, to treat different pathologies, mainly several types of oncological diseases [3]. However, there is still much to understand about protein kinases' roles in health and disease. For instance, protein kinases linked with apoptosis pathways have emerged as promising targets for neurodegenerative diseases [4,5]. A major challenge for upcoming protein kinase inhibitors will be to display outstanding selectivity and efficient brain penetrance opening the door for immunological and neurological disorders [6].

Mitogen-activated protein kinases (MAPKs) are a group of kinase superfamily involved in inflammation processes, innate immunity, apoptosis, proliferation, and differentiation [7,8]. MAPK-regulated signalling pathways play a key role in several diseases [9], including cancer and neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS).

Among the different MAPKs, three categories have been distinguished in mammals. The first one, c-Jun NH_2-terminal kinases (JNKs) which regulate gene expression, neuronal plasticity, regeneration, apoptosis, or cellular senescence [10]. Secondly, p38 MAPKs are related with cellular activities in the central nervous system (CNS) such as proliferation, differentiation, survival, and stress-induced apoptosis [11]. Lastly, extracellular signal-regulated kinases (ERKs) have been described in different studies as downstream activators of cytoplasmic proteins such as mammalian target of rapamycin (mTOR), which is related to homeostasis processes by influencing transcription, translation, and cellular autophagy. ERK is a key element in neuroinflammation which is directly associated with AD and other neurodegenerative diseases [12].

The MAPK pathways mediate intracellular signalling in response to a

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ABBREVIATURES

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
APP	amyloid precursor protein
ASK	apoptosis signal-regulating kinase
CADPS2	Calcium-dependent activator protein for secretion 2
CD36	cluster of differentiation 36
Cdc 42	cell division cycle 42
CDK5	cyclin-dependent kinase
COX-2	cyclooxygenase-2
CPNE8	copine VIII
CRIB	Cdc42/Rac interacting binding
CypD	cyclophilin D
DHN	dihydronaphthyl[3,4-*a*]pyrrolo[3,4-*c*]carbazole
DJ-1	protein deglycase or Parkinson disease protein 7
DLK	dual leucine zipper-bearing kinase
DLP	dynamin-like protein
ERK	extracellular signal-regulated kinase
FUS,	fused in sarcoma RNA binding protein
GSK	glycogen synthase kinase
IGF-1	insulin growth factor 1
iPSC	induced pluripotent stem cells
JNK	c-Jun NH2-terminal kinase
LRRK2	leucine-rich repeat serine/threonine-protein kinase 2
LZK	leucine zipper-bearing kinase
MAPK	mitogen-activated protein kinase
MEK	mitogen activated protein kinase kinase or MAP2K
MLK	mixed lineage kinase
Mnf2	mitofusin-2
MPTP	1-methyl-4-phenyl-tetrahydropyridine;
mTOR	mammalian target of rapamycin
PAK	p21-activated kinase
PARK7	Parkinsonism associated deglycase kinase
PD	Parkinson's disease
PDB	protein data bank
PGE2	prostaglandin E2
PHD	plant homo domain
PINK1	PTEN induced kinase
PKA	protein kinase A
PKC	protein kinase C
PRKN	Parkin or E3 ubiquitin protein ligase parkin
PS1	protein presenilin-1
PTEN	phosphatase and tensin homolog
RAGE	receptor for advanced glycated end products
RTK	receptor tyrosine kinase
SH3	Src homology 3
SNCA	α-synuclein mutated protein
SNpc	substantia nigra pars compacta
SOD	superoxide dismutase
TAK1	transforming growth factor beta-activated kinase 1
TDP-43	TAR DNA-binding protein
TLR-2/4	Toll like receptors
TNF	tumour necrosis factor
ZAK	leucine-zipper and sterile alpha motif kinase

wide number of external stimuli, in which at least three kinase families are involved as a multi-enzymatic cascade: MAPK family, MAPK kinase (MAP2K) family and MAPK kinase kinase (MAP3K) family. Firstly, MAP3Ks are activated by phosphorylation in the cytosol in response to extracellular stimuli. Next, MAP3Ks phosphorylate two activation loop Ser/Thr residues on MAP2Ks. Then, MAP2Ks proceed with the dual phosphorylation of Tyr and Ser/Thr residues in MAPKs. This dual specificity of MAP2Ks is suggested to be a regulatory mechanism for setting up the order of the phosphorylation cascade [13]. Lastly, MAPKs phosphorylate either cytoplasmatic targets or transcriptional factors in the Nucleus [14]. This turns MAP3Ks into important upstream regulators of MAPKs (Fig. 1). Among the MAP3Ks, we can find the mixed-linage kinases (MLKs). MLK family is comprised of three groups: MLKs (MLK1, MLK2, MLK3, MLK4), dual leucine zipper-bearing kinases (DLKs), and the leucine-zipper and sterile-alpha motif kinase (ZAKs) (Fig. 2) [15].

MLKs as potential targets for neurodegenerative diseases treatment have been explored both through pharmacological modulation and genetic approaches. Knock-out mice lacking either *Mlk1*, *Mlk2*, or both, are viable, fertile, healthy, and have a normal life span, despite their important role as upstream regulators of MAPKs [16]. Similarly, mice that have targeted disruption of the *Mlk3* gene had no negative phenotypes [17]. This seems to point towards a functional redundancy within the MLK subfamily. Compensatory mechanisms are a mayor limitation

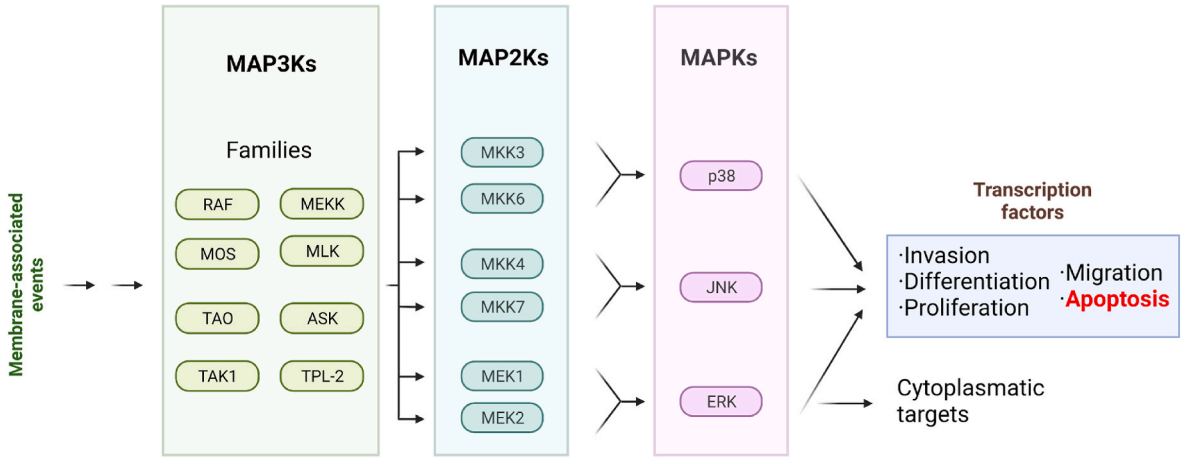


Fig. 1. MAPKs signalling pathways. MAP3Ks are classified into 8 families. Upon activation, they phosphorylate Tyr and Ser/Thr activation loops of MAP2Ks. Activated MAP2Ks proceed with the MAPKs activation through phosphorylation. Lastly, MAPKs travel to the nucleus for transcription factors phosphorylation. MAPKs are responsible for several cellular processes such as invasion, differentiation, proliferation, migration, and apoptosis. Some MAPKs also phosphorylate cytoplasmatic targets.

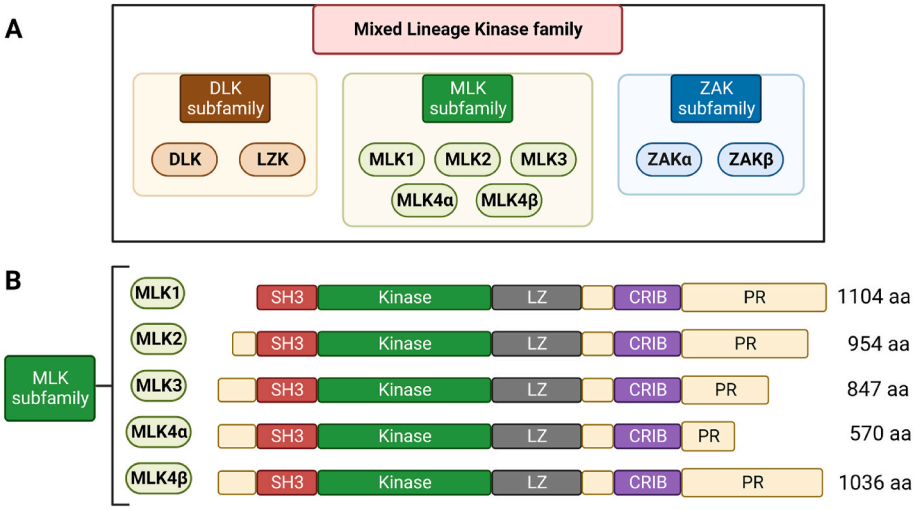


Fig. 2. A) The Mixed Lineage Kinase family divides into 3 subfamilies: DLK subfamily (DLK and LZK), MLK subfamily (MLK1, MLK2, MLK3, MLK4α, and MLK4β), and ZAK subfamily (ZAKα and ZAKβ). B) The structural domains within the MLK subfamily are highly conserved. They all retain the Src homology 3 (SH3) domain, the catalytic kinase domain, the leucine-zipper (LZ) domain (which involves two LZ subdomains with a spacer in between), and Cdc42- and Rac-interactive binding (CRIB) domain. The major structural difference is found in the proline rich (PR) C-terminal tail. The PR region most likely induces somewhat selectivity for the kinase's targets.

when it comes to knock-out genes. It can lead to genetic compensation by expressing homologous proteins to replace the activity of the unexpressed target. Masking the protein of interest's functionality generates false negatives in target validation. As a result, MLKs knock-outs suggest they are not adequate targets. Alternatively, pharmacological modulation can bring more insight and accuracy into the therapeutic potential of MLKs. In fact, one MLK inhibitor, CEP-1347, reached clinical trials based on their safety profile and *in vitro* and *in vivo* neuroprotective properties [18].

Among the four MLK members, MLK3 is the most widely expressed. MLK3 activates not only MAP2K family members, but also their downstream MAPK members, JNK, p38, and ERK [19]. Thus, MLK3 is an exceptionally interesting target to deal with neurodegenerative diseases [20], due to its unique role in stress-mediated neuronal cell death. MLK3 has also been associated with cancer and inflammation, which has been studied and reviewed elsewhere [21]. This review will focus on the role of MLK3 in neuron-related cell death, neurodegenerative diseases, and the potential advantages of targeting this upstream regulator of MAPKs for neuroprotective treatment.

2. MLK3: isoforms, structure and biological neuronal role

MLKs comprise different isoforms with different expression patterns and specific roles. MLK1 (MAP3K9), is expressed in the brain, epithelial tumour cell lines of colonic, breast, and esophageal origin [22]. MLK2 (MAP3K10) is expressed in the skeletal muscle and brain [23]. MLK3 (MAP3K11) is broadly distributed throughout the body [24] and MLK4α and 4β (MAP3K21) mainly in human pancreas and kidneys [25]. Structurally, the protein domains of the different MLK subfamily are highly conserved. The domain architecture consists of an N-terminal Src homology 3 (SH3) domain, which is essential for the enzyme regulation and autoinhibition; a catalytic kinase domain; a tandem Leu/Ile zipper (LZ) domain and a Cdc42/Rac interacting binding (CRIB) domain. LZ domain plays a pivotal role in the activation of MLK3 as it enables

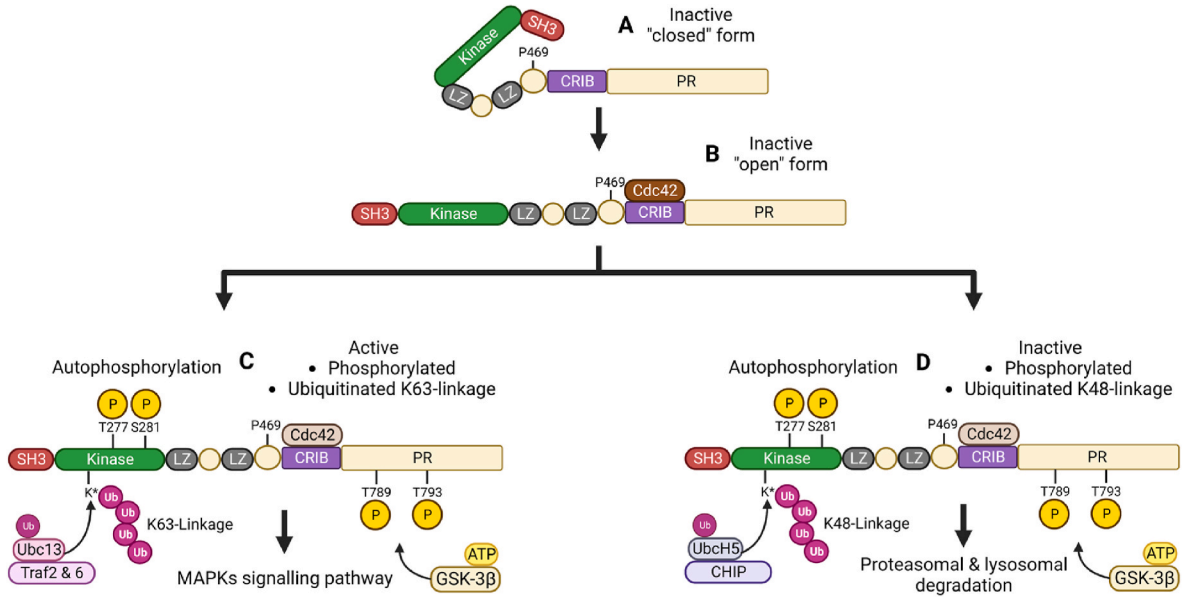


Fig. 3. Activation and degradation mechanisms for MLK3 in neurodegenerative diseases. A) Natively, MLK3 displays a closed conformation due to the interactions between SH3 domain and P469. B) Cdc42 interacts with CRIB domain inducing conformational changes leading to the open form. C) GSK-3β phosphorylates MLK3 at T277 and S281. Traf2&6 E3s with Ubc13 E2 incorporate K63-linked polyubiquitin. The dual PTMs prompt MLK3 active state which initiates MAPKs signalling pathway. D) In contrast, CHIP E3 with UbcH5a E2 incorporate K48-linked polyubiquitin which leads to proteasomal and lysosomal degradation of MLK3. The lysine (K*) positions for MLK3 ubiquitination have not been identified yet.

homodimerization [26,27]. Lastly, there is a region with variable length in each family member rich in Pro, Ser and Thr (Fig. 2).

Currently, only the crystal structures of SH3 domain of MLK3 (PDB ID: 5K28, 5K26) [28], the kinase domain of MLK1 (PDB ID: 3DTC) [29] and MLK4 (PDB ID: 4UY9) [30] have been solved. Natively, MLK3 displays a closed (inactive) quaternary structure facilitated by a single proline residue at position 469, located within the LZ and the CRIB motif, which binds to the SH3 region (Fig. 3A and B). Site-directed mutagenesis of this proline generates an increased catalytic activity, suggesting that this interaction is fundamental for maintaining the inactive state. This critical proline is present in MLK1 and MLK2 suggesting that this regulatory mechanism is conserved within the MLK subfamily [31].

At cellular level, MLK3 is activated by metabolic stress, reactive oxygen species (ROS) and the tumour necrosis factor-α (TNF-α) [32,33]. Activation is mediated by GTP-bound Rac or Cdc42 by interaction with the CRIB domain [26]. This results in conformational changes disrupting the SH3 domain and P469 binding. The "open" state facilitates both phosphorylation and ubiquitination. Autophosphorylation takes place at T277 and S281 within the activation loop [27,34]. Interestingly, GSK3-Beta phosphorylates S789 and S793 upon NGF withdrawal [35]. Also, phosphorylation was reported at S705 and S758 by CDK1 & CDK2 during cell division [36]. Hence, activation loop phosphorylation is critical but additional sites seem to be cell type and disease dependent. TNF receptor associated factor (TRAF) 2 and 6 are Really Interesting New Gene (RING) E3s responsible for the K63-ubiquitination [37]. As RING-type E3s, they require ubiquitin conjugating enzymes (E2s) with direct-lysine ubiquitination activity such as Ubc13 [37]. These dual PTMs facilitate MLK3 dimerization for the active form of the kinase. In contrast, carboxyl terminus of Hsc70-interacting protein (CHIP) a U-box type E3 together with the E2 UbcH5 mediates K48-ubiquitination for inducing proteasomal and lysosomal degradation [38] (Fig. 3D). To sum up, K63-ubiquitination, and autophosphorylation at T277 and S281 induce the active form of the kinase. Whereas K48-ubiquitination is responsible for regulating endogenous levels of MLK3.

Neurodegeneration in cells is triggered by four mechanisms: ubiquitin-proteasome-autophagy system, mitochondrial dysfunction, endoplasmatic reticulum (ER) stress, among others [39]. In normal conditions, ER stress induces unfolded protein response (UPR) as an adaptative reaction for restoring cellular protein homeostasis. UPR regulates protein folding, degradation pathways and induce apoptosis upon irreversible damage [40]. Abnormal ER-stress alter UPR programming leading to high levels of protein misfolding. Aggregation of misfolded proteins is a common hallmark for neurodegenerative diseases [41]. Their accumulation is toxic for the cells, deteriorating their functioning, which triggers apoptosis through MAPKs pathway. Additionally, altered UPR also induces apoptosis by directly upregulating the MAPKs pathways [42].

Note that unlike other cells, neurons are typically incapable of regeneration. Thus, overactivation of cell-death-related signalling pathways leads to irreversible neuron loss. Neuroprotection has been demonstrated when induced apoptosis has been interrupted by inhibiting kinases like MLK1, MLK3, p38 or JNK present within the stress signalling pathways [43]. Among the MLK family, MLK3 is responsible for most of the of MAPKs activation [44]. Since it has been strongly linked to ERK, p38, or JNK activation, the inhibition of MLK3 has the potential to avoid the activation of these three MAPKs simultaneously. As consequence, transcriptional factors and caspase pathways are diminished. In fact, pharmacological modulation of MLK3 has proven to exert neuroprotection in several cell lines as showed in the next sections.

3. MLK3 in neurodegenerative diseases

Neurodegenerative diseases are characterized by the progressive cellular loss of the central nervous system. The ethology is disease-dependent for all of them being complex diseases where many cellular pathways and different proteins are disturbed [45]. Drug discovery programmes have been tempered by the several challenges of neurodegenerative diseases. For instance, there is an urge in developing new tools for target validation as for most of these diseases it is still unclear the most suitable targets to address. In fact, to date only some palliative treatments have been approved with limited efficacy among the patients. Neuroprotective strategies that avoid or delay the neuronal loss are suggested to be potential therapies to alleviate degeneration. MLK3 modulation arises as a potential therapy for neurodegenerative diseases prolonging the quality of patient's life.

4. Alzheimer's disease

Alzheimer's disease (AD) is characterized by neurodegeneration, neural loss, and development of neurofibrillary tangles and amyloid-β peptide (Aβ) plaques, without any effective treatment up to the moment [46]. The presence of Aβ plaques leads to oxidative stress and subsequent activation of p38 pathway [47]. Furthermore, MLK3-MKK/7-JNK3 cascade is activated by Aβ aggregates. In fact, JNK3 has been extensively related to AD and the mechanisms by which JNK3 induces cell death in AD reviewed elsewhere [48]. Aβ has been shown to increase GSK3β activity [49], and GSK3β has been proven to directly phosphorylate MLK3 at Ser789 and Ser793 [35]. As described, MLK3 activation triggers the MAPK-dependent cell death pathways. Pharmacological modulation of MLK3 by K252a ligand have proven to reduce Aβ-induced apoptosis (Fig. 4A) [50].

Additionally, microglia could perform a significant role in AD and neuronal cell death. Microglia interaction with Aβ aggregates triggers two different responses: endocytosis followed by clearance or production of cytokines and ROS that induce p38 pathway activation in neighbouring neurons [51,52]. Natural Aβ aggregates clearance is mediated by microglia transmembrane protein SR-A (scavenger receptor A) [53]. Nevertheless, such activity is not sufficient to prevent AD progression. Additionally, microglial transmembrane proteins such as RAGE (receptor for advanced glycated end product) [54], CD36 and TLR-2/4 (Toll like receptors 2/4) [55], are activated by Aβ aggregates which leads to p38 pathway activation (Fig. 4B). Thus, tackling MAPKs upstream activators such as MLK3 could be an interesting approach to prevent direct and indirect neuron loss in AD caused by Aβ and microglia-mediated respectively.

Together with Aβ, tau tangles are hallmarks of AD. Tau protein plays a major role in microtubules stabilization and avoidance of undesirable depolymerization [56]. Microtubule-associated protein tau undergoes extensive hyperphosphorylation and ubiquitination. Phosphorylation is mediated by GSK3β [57], cyclin-dependent kinase 5 (CDK5) [58] and MAPKs [59,60] among other kinases (Fig. 4C). These aberrant PTMs on tau triggers the formation of instability, making the neurons more prone to suffer physical cellular damage [61]. Unlike GSK3β, MLK3 has not been proven to directly interact with tau. However, as it was previously mentioned, GSK3β can directly phosphorylate MLK3, which increased neuronal cell death in nerve growth factor (NGF)-withdrawal PC12 cells [35]. Because tau phosphorylation/aggregation and MLK3 activation do not happen independently from each other, they should not be studied individually. Further research is needed to elucidate how these important hallmarks impact each other and influence cellular fate in AD.

On the other hand, mitochondrial dysfunction has a key role in neurodegenerative diseases [62]. In AD it has been related to the binding of Aβ aggregates with membrane protein cyclophilin (CypD) [63]. Such binding blocks the protein functionality, leading to mitochondria swelling followed by oxidative stress that ultimately triggers p38 pathway. Interestingly, a study involving *cybrids* (cytoplasmic hybrids, a model to study mitochondriopathies), demonstrated the implications of dynamin-like protein 1 (DLP1) and mitofusin-2 (Mnf2) in AD. Healthy cells had their mitochondria substituted by mitochondria of AD patients to study the dysregulations generated by the unhealthy mitochondria. Morphological and functional changes were detected in DLP1 and Mfn2.

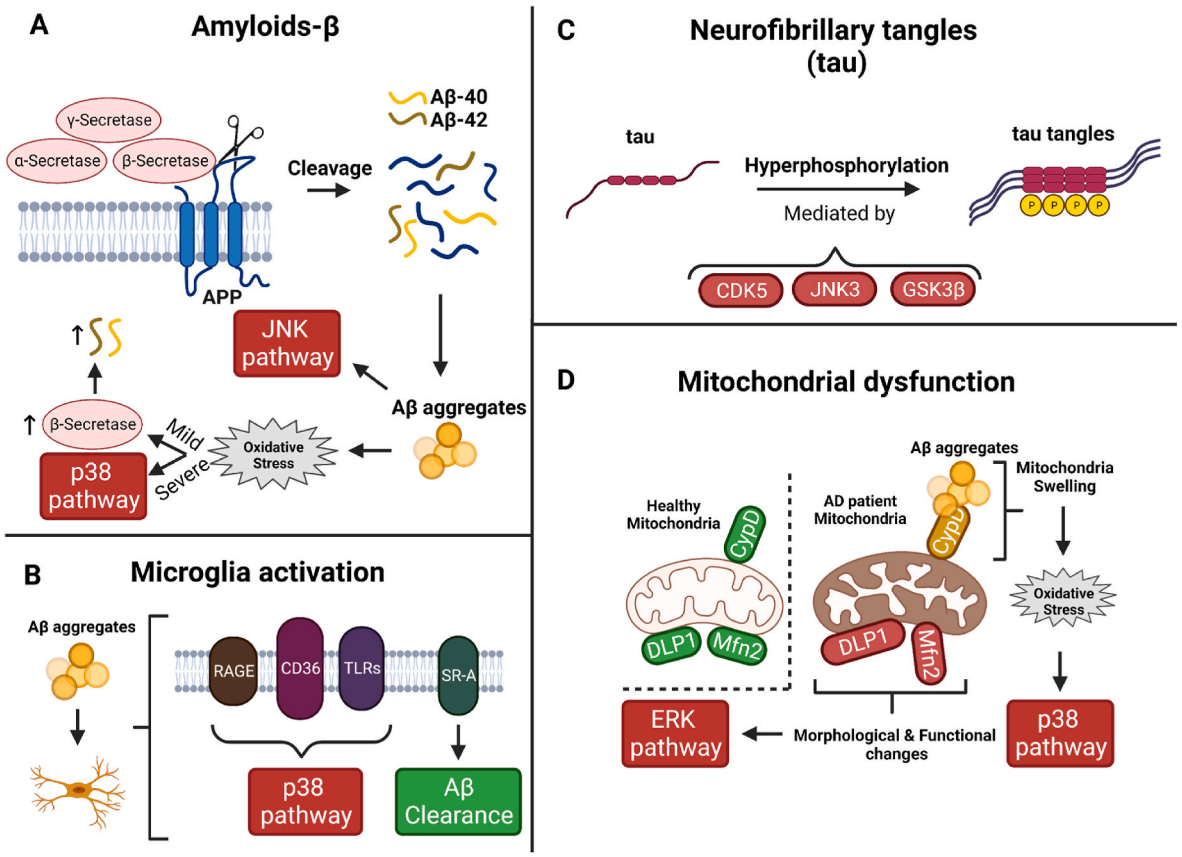


Fig. 4. Alzheimer's disease pathological hallmarks: Amyloid-β, microglia activation, neurofibrillary tangles, and mitochondrial dysfunction. Upon progression, MAPKs (p38, JNK, and ERK) signalling pathways get upregulated leading to neuron loss.

Treatment with ERK inhibitors recovered the healthy state for both DLP1 and Mfn2 [64] (Fig. 4D). As ERK-MLK3 cascade activation pathway was already discussed earlier in this review, upstream modulation would have similar impact as ERK inhibitors. Thus, MLK3 plays a dual role in AD: direct MAPKs activation and signalling amplification. MLK3 modulation prevents cell-death signalling suggesting therapeutic potential for AD. Given the numerous AD-related targets in the disease network, only candidates with wide biological neuroprotective effect such as MLK3 inhibitors or multi-target-directed ligands are considered as promising strategy to treat AD having started the first clinical trials [65].

5. Parkinson's disease

In Parkinson's disease (PD), nigral dopaminergic neuron loss occurs upon misfolding of certain neural proteins. Several genes such as *SNCA* (α-Synuclein mutated), *LRRK2* (leucine rich repeat kinase 2), *DJ-1* (PARK7), *PINK1* (Phosphatase and tensin homolog (PTEN)-induced kinase), *Parkin* and *HtrA2/Omi* (high temperature requirement A2/Omi) mutations have been reported in PD patients [66].

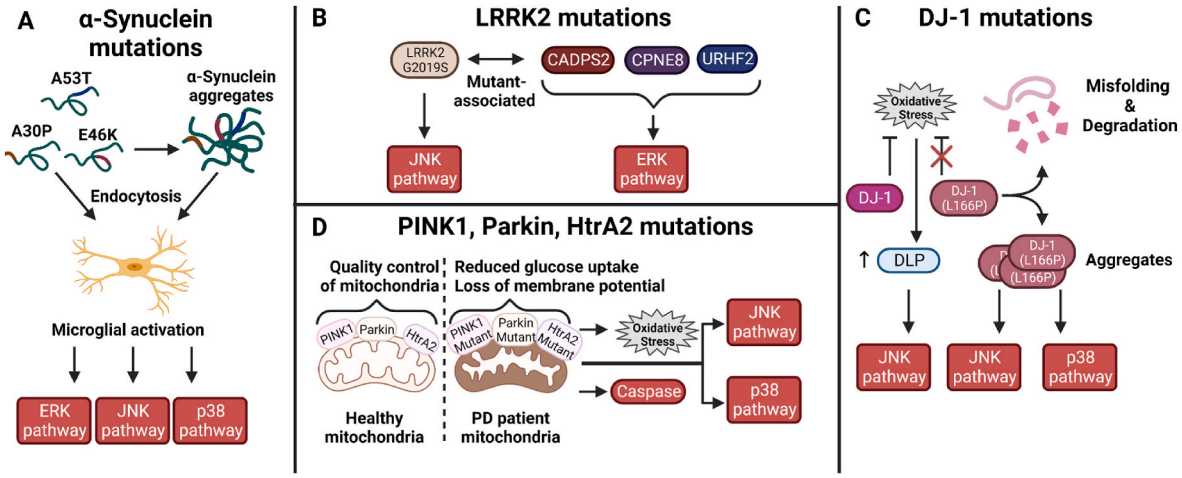


Fig. 5. Parkinson's disease pathological hallmarks: α-synuclein, LRRK2, PINK1, Parkin, HtrA2 and DJ-1 defects. Mutated genes lead to activation of MAPKs (p38, JNK, and ERK) signalling pathways which ultimately leads to neuron loss.

The protein α-synuclein has a key role in the pathogenesis of the PD, although the exact mechanism producing toxicity and neuronal death is still unclear [67]. Previous studies have shown that α-synuclein regulates the MAPK pathway by reducing the amount of available active MAPK although further study will be necessary for the effect of α-synuclein aggregates on MAPK phosphorylation [68] (Fig. 5A). These findings suggest a mechanism for pathogenesis and thus offer therapeutic insight into synucleinopathies.

LRRK2 is one of the most often mutated genes in PD patients [69]. Particularly, LRRK2^{G2019S} has been noted to be the most frequent mutation for LRRK2 [70], and it has been reported the potential implication of *LRRK2* mutants as inducers of α-synuclein aggregates [71]. Studies on induced pluripotent stem cells (iPSC) from PD patients have shown that LRRK2 mutation is accompanied by dysregulations on genes *CADPS2* (Calcium-dependent activator protein for secretion 2), *CPNE8* (Copine VIII), and *URHF-2* (Ubiquitin-like with PHD and ring finger domains 2). All of them are related to dopaminergic neuron loss via ERK pathway [72] (Fig. 5B). LRRK2^{G2019S} is able also to activate MKK4-JNK3 pathways inducing dopaminergic neuron loss in mouse models [73]. MLK3, being a specific MKK4 modulator, may be a good target to alleviate the cell death induced by LRRK2 in the progression of PD.

DJ-1 is a protein exerts protective behaviour against oxidative stress [74]. The mutated gene *L166P* leads to the expression of a variant with a lack of such functionality. It tends to misfold, be degraded, and even aggregate, in which case JNK and p38 activation have been reported [75]. Additionally, the lack of functionality of DJ-1 leads to no mitigation of oxidative stress in *Drosophila* that ultimately activates JNK pathway [76] (Fig. 5C). Likewise, growth factor deficiency in PD patients' brains is thought to be relevant in PD onset and progression [77]. It has already been mentioned how NGF withdrawal can activate MLK3. So, it is not far-fetched to think that this deficiency may cause GSK3β-MLK3-JNK activation and dopaminergic cellular death [35].

On the other hand, mitochondrial dysfunction in PD has been shown to play a pivotal role in the development of the disease [78]. It has been associated with three genes: *PINK1*, *PARKIN* [79], and *HtrA2/Omi* [80] mutants have shown to reduce glucose uptake and loss of membrane potential, generating oxidative stress and caspase activation. JNK3 and p38 pathways activation has been proven due to dysregulations generated by the mutated proteins [9] (Fig. 5D).

JNK activation has been induced in mammalian cells through MLK3 transfection showcasing the modulation of dopaminergic neuronal cell death [81]. CLFB-1134, a MLK3 inhibitor, have been proven to exert protection against MPTP (1-methyl-4-phenyl-tetrahydropyridine)-induced dopaminergic neuron loss by avoiding JNK3 phosphorylation [82]. 6-hydroxydopamine (6-OHDA) is another neurotoxin used for inducing neuron less through JNK signalling pathway. Upon treatment with K252a, it was prevented through MLK3 inhibition which downregulated JNK pathway [83]. Thus, the important milestone of evaluating the efficacy of a MLK3 inhibitors for PD in clinical trials, detailed in the next section for CEP-1347 [18], has already been achieved, setting a precedent for further investigation.

6. Amyotrophic lateral sclerosis

On a cellular level, protein aggregates composed of TAR DNA-binding protein (TDP-43), superoxide dismutase 1 (SOD1) and fused in sarcoma RNA binding protein (FUS) are commonly found in amyotrophic lateral sclerosis (ALS) motor neurons [84]. However, ALS aetiology remains weakly understood. 90% of the cases are considered sporadic ALS caused by undetermined environmental factors. Less frequently, mutations in the genes coding for SOD1, TDP-43, and FUS can be blamed for the malfunction and aggregation of these proteins [85].

Among the several mutants studied for SOD1, two have shown relevant connections with ALS pathology: SOD1^{G85R} and SOD1^{G93A} [86]. SOD1^{G85R} has been reported to inhibit anterograde axonal transport leading to phosphorylation of p38 and subsequent activation of this pathway [87]. Moreover, SOD1^{G93A} mutant has been shown to activate p38 pathway as well [88] (Fig. 6A). Furthermore, FUS misfolding has been also linked with aberrant p38 activation, with increased levels of p38 in post-mortem analysis in humans with FUS-induced ALS [89] (Fig. 6C). Therefore, all these alterations converge in MAPKs activation, especially p38, promoting cell death and neuron loss. How ALS is influenced by JNK/p38/ERK aberrant hyperactivation and hyperphosphorylation has been already reviewed somewhere else [90]. Still, it is important to note that both JNK and p38 downregulation has been linked to extended survivability in SOD1-mutant ALS mice. Also, insulin growth factor 1 (IGF-1) has shown to decrease the levels of activated (phosphorylated) p38 and JNK, hence increasing mice survivability [91].

Lastly, neuroinflammation has been key to completing the understanding of ALS. It has been observed to be primarily related to microglia and astrocytes [92]. Moreover, both types of glial cells have been directly implicated in SOD1-mediated neurodegeneration in ALS [93]. Cyclooxygenase-2 (COX-2), a key protein in inflammatory response, has been studied in relation to aberrant TDP-43, ALS, and neuroinflammation. COX-2 levels are upregulated in the brain and spinal cord of post-mortem ALS patients and in mouse models [94]. COX-2 and prostaglandin E2 (PGE2) production has been linked to MAPKs/ERK pathway activation. Upon pathological loss of TDP-43 function in microglia, the ERK pathway is upregulated in this type of cell. Thus, PGE2 has been proposed as a novel molecular mediator of indirect TDP-43 neurotoxicity. This has been supported by the fact that celecoxib, a COX-2 selective inhibitor, has been demonstrated to display neuroprotection and prolonged neuron survivability [95] (Fig. 6B).

Overall, MLK3 has links to the three major neurodegenerative diseases. While not being subject of direct genetic mutations or taking part of the aetiology of neither of them, it is involved in the pathology development, and it serves as an effector of the irreversible neuron loss. Cell death-induced pathways mediated by JNK, p38, and ERK (MAPKs) can be simultaneously addressed through the pharmacological modulation of MLK3. Ultimately, the scope is to slow down aberrant ERK-stress cascade enzymes. This can proof to be a novel and efficient strategy to address neurodegenerative diseases. Studies aimed to modulate MLKs for the discovery of new neuroprotective therapies should be further explored.

7. MLK3 inhibitors

Developing selective compounds for protein isoforms is a major challenge in drug discovery. The MLK subfamily is no exception, yet we have compiled a series of reported ligands designed to target MLK1, MLK2 and MLK3. Despite the difficulties, new hits have progressively arisen over the years with increased selectivity and enhanced pharmacological properties (Table 1).

The first reported MLK inhibitor was the glycosilated alkaloid K252a, isolated from *Nocardiopsis* spp [99]. This natural product, analogue to staurosporine, was initially evaluated as inhibitor of protein kinase C. Subsequent studies demonstrated its properties as a non-specific protein kinase inhibitor [100]. Later on, K252a was reported as a potent MLK3 inhibitor with an $IC_{50} = 5$ nM [96] and was used for *in vitro* studies addressing neurodegenerative diseases, such as AD and PD.

In the context of AD, the neurotrophic properties of K252a and staurosporine derivatives are well known [101]. The protection of cultured rat hippocampal neurons after the insult with Aβ and treatment with K252a has been reported [102]. More recently, it was demonstrated the activation of the MLK3 signalling pathway due to Aβ toxicity and the neuroprotective effect of treatment with K252a [50]. These findings show not only the important role of the MLK3-MKK7-JNK3 in the mechanisms underlying AD neurodegeneration, but also that K-252a and related compounds can be useful drugs to stop the progressive neuronal death produced by one of the hallmarks of AD.

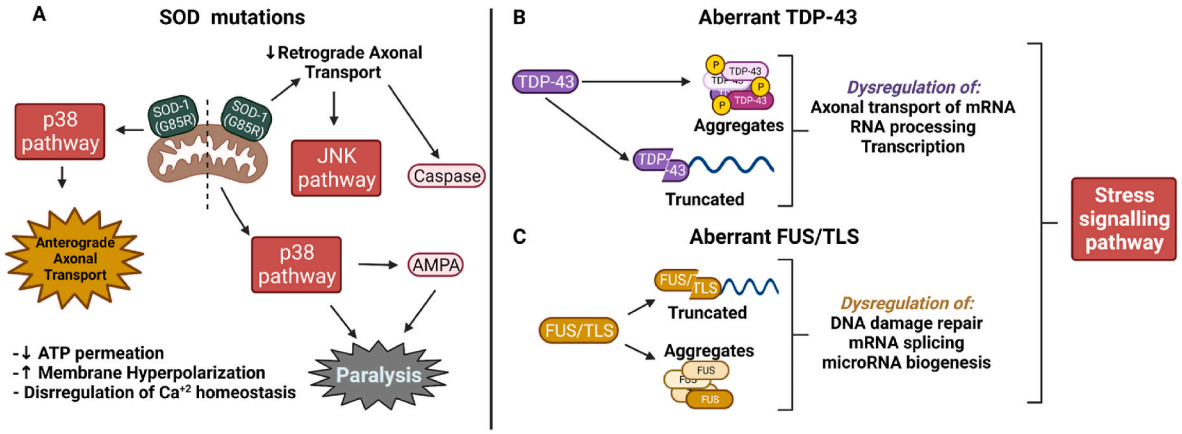
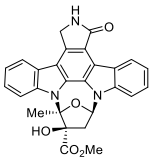
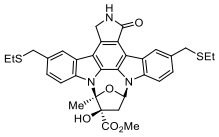
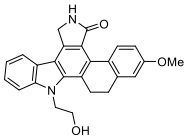
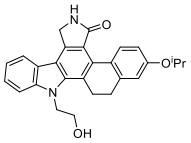
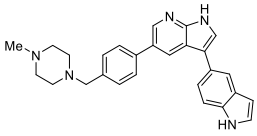
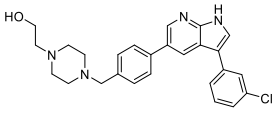
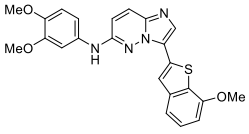


Fig. 6. Amyotrophic lateral sclerosis pathological hallmarks: SOD-1 mutations, TDP-43 and FUS/TLS aggregates/truncations. Indicated mutations and aggregates lead to the activation of the stress signalling pathways (p38, JNK, and ERK).

Table 1
Reported MLK inhibitors and enzymatic activities in MLKs.

Compound	Chemical structure	MLK1 (IC_{50} nM)	MLK2 (IC_{50} nM)	MLK3 (IC_{50} nM)
K-252a [96]		Not reported	Not reported	5
CEP-1347 [97]		38 ± 17	51 ± 9	23 ± 0.1
DHN-14 [29]		103 ± 20	1582 ± 46	55 ± 17
DHN-16 [29]		26 ± 12	1855 ± 87	67 ± 9
URMC-099 [98]		19	42	14
Prostetin/12k [43]		44.7	Not reported	23.7
CFLB-1134 [82]		Not reported	Not reported	Not reported

With respect to PD, it is well known the role of MAPK pathway in rats with 6-OHDA-induced lesion in the nigrostriatal system. Substantia nigra *pars compacta* (SNpc) tissue was examined after treatment with 6-OHDA toxin followed by K252a treatment. The analysis confirmed the reduction of apoptosis via the programmed signalling pathway of MLK3. Control treated only with 6-OHDA showed marked nigral neuron death. The inhibition effects of K252a were confirmed by immunoblot analysis of phospho-MLK3 and anti-MLK3. The findings suggested that K252a could protect neurons from 6-OHDA-induced damage having interest as therapeutic agent for PD [83].

The selectivity of this drug candidate is very poor as it displays inhibition profiles towards other kinases, such as Trk, PKC, PKA, CDKs [103–105]. Due to this fact, research has been conducted on the design of derivatives with higher selectivity, especially for the PAK family. Interestingly, such studies led to the synthesis of derivatives such as CEP-1347, which bears an indolecarbazole scaffold as K252a, and an (ethylthio)methyl substituent in positions 3 and 9. Remarkably, the selectivity was indeed improved towards PAK but also MLK family [97]. CEP-1347 stands out for its relevance, since it reached clinical trials as a disease-modifying therapy for PD [18].

As CEP-1347 interferes with MLK family activity [97,106], in human neuroblastoma SH-SY5Y cells, treatment with the drug suppressed MPTP-induced cell death by disrupting the JNK signalling pathway. CEP-1347's capacity to protect SNpc dopamine neurons from MPTP toxicity has been shown in mice [106,107], but also in primates. MPTP was administered to cynomolgus monkeys on a weekly basis, and those given CEP-1347 had less parkinsonism than those given vehicle [108].

Previous mentioned efficacy studies, along with proven safety and tolerability in 30 patients with PD [109] were the basis for proceeding with larger clinical trials in order to determine CEP-1347 neuroprotective capacity in PD patients. Named PRECEPT, the trial enrolled 806 participants with early-stage Parkinson's disease [110]. Despite failure of PRECEPT, the interpretation of gathered data do not provide decisive evidence of MLK inhibition as a wrong target for neuroprotection. As the relationship between neuronal loss and PD progression is yet to be fully described, it can lead to misinterpretations and mistaken assessments of neuroprotective approaches [111]. On the other hand, it is possible that CEP-1347 did not effectively inhibit JNK activity through MLK inhibition due to limited pharmacokinetic properties that prevent the drug from crossing the blood-brain barrier and prevent the availability of CEP-1347 in brain tissue [98].

Although unsuccessful clinical trials, the potential of CEP-1347 as a neuroprotective agent toward dopaminergic neuron loss via MLK family inhibition drove further research on the design of novel derivatives with improved properties. In 2008, new derivatives inspired by indolocarbazoles CEP-1347 and K252a were reported [29,112]. Two modifications were set in the main scaffold: Elimination of the tetrahydrofurane moiety and substitution of one of the pyrrole rings by a cyclohexyl ring, which results in the dihydronaphthyl[3,4-*a*]pyrrolo[3,4-*c*]carbazole (DHN) scaffold. Derivatives, DHN-14 and DHN-16 were subsequently designed by optimization of positions R^2 and R^{12}, showing MLK1 and MLK3 subtype-selectivity within the MLK family.

Moreover, the crystal structure of the complex MLK1 - DHN-16 was solved to identify the most relevant interactions, which ultimately help further molecule design [29]. Additionally, the kinase selectivity profile was examined for a set of compounds [29]. CEP-1347 shows activity towards MLK1, MLK2, MLK3, and DLK, among other kinases, whereas newly reported compounds showed to success in this aspect, as selectivity was improved towards MLK1 and MLK3. Thus, DHN-14 displayed selectivity for MLK1 (IC_{50} = 103 nM) and MLK3 (IC_{50} = 55 nM), as also DHN-16 in MLK1 (IC_{50} = 26 nM) and MLK3 (IC_{50} = 67 nM). Since the pan-MLK inhibitor CEP-1347 displays neuroprotective effects in MPTP induced models of PD *in vitro* and *in vivo* [108], the improved selectivity profile of DHN derivatives motivated their evaluation in cellular and mice MPTP models [29]. As a result, both tested compounds generated a substantial reduction in dopaminergic neuron loss. The higher selectivity profile of the derivatives towards MLK1/3 allowed to confirm the neuroprotective effect of inhibition of those two kinases.

A new family of potent MLK3 inhibitors was reported in 2013 bearing a 7-azaindole scaffold [98]. The active compounds were identified after a high-throughput screening campaign using a library with 15000 compounds. Subsequent efforts for hit to lead optimization resulted in a family of azaindole derivatives. After a thorough study in search of optimal solubility-absorption properties, the derivative URMC-099 was reached as the optimized candidate. 7-Azaindole URMC-099 showed good oral bioavailability, being able to access the CNS although metabolic stability remained clearly improvable. URMC-099 achieved only modest kinase specificity and low selectivity between the more representative isoforms of the MLK family, such as MLK1 (IC_{50} = 19 nM), MLK2 (IC_{50} = 42 nM), MLK3 (IC_{50} = 14 nM), and a good profile for the related MLK family member DLK (IC_{50} = 150 nM).

Different biological studies using URMC-099 were performed to determine the mechanism of action of this drug candidate in the context of neurodegenerative diseases. It was reported a partial protection from Aβ-mediated microglial cytotoxicity, by reduction in phosphorylation of MKK3/MKK4 and p38/JNK, suggesting that the therapeutic effect of URMC-099 is mediated by targeting the MLK pathways in Aβ-mediated neuroinflammation in microglia. Thus, URMC-099 could be considered as a neuroprotective agent to be used as therapeutics for AD [113]. This assertion was reinforced by the fact that URMC-099 facilitates Aβ clearance in the brain of APP/PS1 mice after i.p. administration at 10 mg/kg daily for 3 weeks [114]. Furthermore, URMC-099 shown hippocampal synapsis neuroprotection in an *in vivo* model of multiple sclerosis [115].

Further structural modifications of URMC-099 led to Prostetin/12k showing the most promising results up to date. It was found a half-life of >120 min in mouse liver microsome assay which is a high improvement compared with URMC-099 which gave a 15 min half-life. Additionally, prostetin/12k had improved brain penetrance compared with URMC-099 [43] which prompted a potent motorneuron neuroprotection. Currently, prostetin/12k has entered in clinical development, NCT05279755 [116], with the goal to treat ALS patients.

The third chemical class of MLK3 inhibitors was reported in 2019. Imidazo[1,2-*b*]pyridazine CFLB-1134 showed effective protection against MPTP-induced dopaminergic neuron loss and was reported to be an specific MLK3 inhibitor, although no data were shown. CLFB-1134 shown a favourable pharmacokinetic profile due to the good brain penetrance. *In vivo* studies confirmed an excellent protection against striatal dopaminergic terminals when this compound was administrated along with or after MPTP injections. However, there was poor effect on dopamine levels in striatal terminals. Lastly, JNK signalling pathway was downregulated by treatment with CLFB-134 through MLK3 inhibition [82].

8. Conclusions and future trends

The lack of effective treatments for neurodegenerative diseases encourages the search of new therapeutic approaches. Protein kinases inhibitors with favourable pharmacokinetic properties and acceptable selectivity profile are required to advance this field further. That is the case of MLK3 inhibitors as wide neuroprotective agents. MLKs inhibitors have proven safe drug response obtained in the clinical trial of CEP-1347, they have been improved in the drug like properties of the new candidates and they have shown good results in different models of neurodegenerative diseases. These paves a new opportunity to reach the clinical setting. In fact, prostetin/12k has recently entered phase I studies with the aim to target ALS patients (NCT05279755) based in its ability to protect motorneurons and cross the blood-brain barriers in preclinical models.

Disease biology within neurodegenerative diseases share certain features such as the presence of different neurotoxic agents, cell metabolism dysregulation, increased ROS levels, and inflammation. They lead to membrane-related events triggering the activation of stress signalling pathways which ultimately induces cell death. Hence, strategies addressing these enzymatic cascades are emerging as potential therapies to prevent neuron loss. In that case, MLK3 inhibitors offer an excellent opportunity as they are able to modulate the three main stress signalling pathways simultaneously. Besides, targeting an upstream kinase increases the chances of diminishing the effect of signal amplification.

Interestingly, a study performed with CEP-1347, a drug candidate for MLK family inhibition reached clinical trials phase II/III. Despite the negative feedback, this trial revealed that the inhibition of MLK3 in the

body did not cause any negative side-effect. That leads to thinking that in normal conditions, other MLK isoforms can replace MLK3 activity. It is important to notice that one common pillar of neurodegenerative diseases (such as AD, PD and ALS) is the overactivation of stress signalling pathways leading to excessive cell damage, apoptosis, and ultimately neuron loss. Hence, disrupting the stress signalling pathway at MLK level could be an interesting therapeutic approach for these common diseases. MLKs, particularly MLK3, may play a significant role in maintaining the high activity of the stress-induced cell death pathways when AD, PD, and ALS pathologies are present.

In the next future, MLK3-specific inhibitors will be discovered as chemical probes to validate unequivocally the therapeutic role of MLK3 in neurodegenerative diseases. Moreover, brain permeable MLK3 specific inhibitors or adequately brain delivered compounds may impact the disease-modifying pharmacological treatment of the above-mentioned devastating diseases. Nanotechnology approaches such as PLGA nanoencapsulation may be also used to overcome the reduced brain penetration [117]. Finally, the lack of an experimental 3D structure for MLK3 hampers the rational drug design of its inhibitors, but recent cryo-EM techniques may also be applied to the resolution of this big protein opening the pathway for speeding the rational design and optimization of MLK3 inhibitors as potent therapeutic agents.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Javier Recio reports financial support was provided by University of Alcala.

Data availability

No data was used for the research described in the article.

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