Abstract: The cdc2-like kinases (CLKs) are an evolutionarily conserved group of dual specificity kinases belonging to the CMGC (cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases). The CLK family consists of four isoforms namely CLK1, CLK2, CLK3 and CLK4. The human CLK1 encoded protein comprises 454 amino acids and the catalytic domain of CLK1 exhibits the typical protein kinase fold. CLK1 has been shown to autophosphorylate on serine, threonine and tyrosine residues and phosphorylates exogenous substrates on serine and threonine residues. CLK1 plays an important role in the regulation of RNA splicing through phosphorylation of members of the serine and arginine-rich (SR) family of splicing factors. CLK1 is involved in the pathophysiology of Alzheimer’s disease by phosphorylating the serine residue in SR proteins. Nuclear speckles of the nucleoplasm contain the stored form of SR proteins and are moderately responsible for the choice of splicing sites during pre-mRNA splicing. Hence, the inhibition of CLK1 can be used as a therapeutic strategy for Alzheimer’s disease. Many natural and synthetic molecules are reported to possess CLK1 inhibitory activity. Some specific examples are Marine alkaloid Leucettamine B and KH-CB19. Leucettamine B is a potent inhibitor of CLK1 (15 nM), Dyrk1A (40 nM), and Dyrk2 (35 nM) and a moderate inhibitor of CLK3 (4.5 µM) whereas KH-CB19 is a highly specific and potent inhibitor of the CLK1/CLK4. X-ray crystallographic studies have revealed that the marine sponge metabolite hymenialdisine and a dichloroindolyl enamine nitrile (KH-CB19) have binding interaction to CLK1. This review focuses on the role of CLKs in the pathophysiology of Alzheimer’s disease and therapeutic potential of targeting CLK1 in Alzheimer’s disease drug discovery and development. In addition, the recent developments in drug discovery efforts targeting human CDC2-like kinase 1 are also highlighted.

Keywords: Cdc2 like kinase (CLK), Alzheimer, SR proteins, protein kinases, CLK1 inhibitors.

INTRODUCTION

Protein kinases are an important family of enzymes consisting of protein tyrosine kinases and serine/threonine kinases. According to the location of the kinases, the protein tyrosine kinases are called receptor protein kinases and cytoplasmic tyrosine kinases [1-4]. These kinases are responsible to control numerous aspects of cell functions, which are exploited in the management of many diseases. The cdc2-like kinases (CLKs) are an evolutionarily conserved group of dual specificity kinases belonging to the CMGC group (cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases, mitogen-activated protein kinases (MAP kinases)) capable of phosphorylating protein substrates on serine, threonine and tyrosine residues [5, 6]. The CLK family consists of four kinases isoforms namely CLK1, CLK2, CLK3 and CLK4 [1]. These four isoforms have a highly conserved domain structure which is located at the C terminus of the molecule. They also have the signature amino acid motif EHLMAMMELG, giving rise to the name of these kinases: LAMMER kinases [1-3] (Fig. 1). The CLK family kinases are found in diverse species, including yeast, Drosophila, Arabidopsis, tobacco, mouse, rat and human beings. The isoforms of CLKs are phosphorylated on serine/threonine and tyrosine residues, (these kinases contain Akt-substrate motifs), thus reveal that the phosphatidylinositol-3-kinase (PI3K)/Akt pathway can regulate the function of CLK [7, 8]. The expression of CLK family enzymes has also been implicated in the regulation of alternative splicing of PKCβII, TF, microtubule-associated protein Tau and α-globin pre-mRNA through the phosphorylation of splicing factors [9, 10]. The overexpression of CLK enzymes localizes to nuclear speckles where splicing factors are concentrated, and it affects splicing site selection of pre-mRNA [11]. In human beings, the highest levels of CLK expression were found in the brain [12, 13].CLK1, also termed STY is a nuclear kinase, initially isolated in a screen designed to identify tyrosine kinases [1, 13, 14]. The physiological role of CLK family kinases is largely unknown in vertebrates, but they may play an important and evolutionarily conserved role in signal transduction within the cell [15]. The critical role of the CLK family kinases in development has been suggested by experimental studies on Drosophila CLK homologue, DOA. Flies expressing low levels of the mutant DOA protein show marked neurologic abnormalities and homozygosity for the DOA null allele is embryonically lethal [15, 16]. Furthermore, studies have also indicated that the CLK family of kinases may have an important function in the regulation of mRNA splicing [15]. Serine/arginine-rich (SR) proteins play essential roles in the constitutive and regulated splicing of precursor mRNAs. CLK1 has been reported to bind and phosphorylate SR proteins in vitro at physiologically relevant sites [17, 18]. Moreover, Colwill et al. has demonstrated that overexpression of CLK1 in COS cells leads to the subcellular redistribution of SR proteins, and to alterations in mRNA splicing in vivo [18, 19]. Collectively, these data strongly suggest a role for the CLK family kinases in the regulation of mRNA splicing in vivo.
Neuronal CLK (NCLK), a heterodimer of a Cdk5 catalytic subunit and a 25 kDa regulatory subunit derives proteolytically from a neuron and central nervous system-specific 35 kDa protein. It is originally identified from bovine brain and it shares common catalytic and regulatory properties with CLK kinase family proteins. This kinase shows critical dependence on the proline residue [20-22]. The peptide phosphorylation by NCLK dependent on the presence of the proline residue on the carboxy terminal to the threonine residue due, peptide analogues with proline residue substituted by glycine or alanine could not be phosphorylated. As a result, the enzyme was originally designated as brain proline-directed protein kinase [21, 23]. NCLK is found in association with Rac and Pak1, further suggesting that the kinase is involved in the reorganization of the actin cytoskeleton in neurons [24].

**STRUCTURE AND FUNCTIONS**

The human CLK1 encoded protein comprises 454 amino acids and the catalytic domain of CLK1 exhibits the typical protein kinase fold (Fig. 1). The N-terminal lobe of the protein incorporates three β strands (β1–β3) followed by a helix (αC) and two additional β strands (β4 and β5). The N-terminus of the CLK1 acts as an anchor to the substrates and also regulates its kinase activity. The N-terminus truncation of CLK1 results in a remarkable increase in CLK1 enzymatic activity, indicating that the N-terminus acts as a negative regulatory domain. An insertion between the sheets β7 and β10 is the most prominent feature observed in the C-terminal lobe. An insertion at the top of the C-terminal lobe where residues 300–317 form an extended β-hairpin structure that packs against a groove formed by helix αD and αE that is a second exclusive insertion conserved in the CLK family [12-13, 25]. The α4 helix contains the LAMMER motif. This suggests that CLK1 is regulated by three different mechanisms that provide both positive and negative regulations of CLK1 activity. CLK1 is positively regulated by the phosphorylation on either tyrosine residues or serine/threonine residues and is negatively regulated by steric constraints mediated by the N-terminal domain, as well as, by phosphorylation on a subset of serine/threonine residues within the catalytic domain [1-3]. The cytoplasmic serine rich protein kinase (SRPK1) phosphorylates the N-terminal stretch of Arg-Ser repeats [(RS)N(S(RS)], termed RS1 in the RS domain of the SR splicing factor (SRSF1). The C-terminal half of the RS domain (RS2) contains a region with several SerPro dipeptides [SPRRSRRGSPRYP] and an additional, shorter Arg-Ser repeat [(SR)s]. In nucleus, the CLK family of kinases catalyzes multisite SR protein phosphorylation on the RS2 domain of SRSF1 [26-28] (Fig. 2).

Studies have shown that overexpression of CLK1 leads to diffused nuclear localization of interchromatin granule clusters which in turn results in accumulation of splicing factors at the sites of pre-mRNA as well as reduced pre-mRNA splicing [12]. The expression of CLK1 in PC12 cells induces the cells to undergo neuronal differentiation which shows that the importance of CLK1 in neuronal development [5]. The ability of CLK1 to differentiate PC12 cells was associated with the CLK1-dependent activation of members of the MAP kinase cascade, suggesting that the function of CLK1 is elicited through signal transduction pathways [5]. The isoforms of CLKs have different functions in virus (HIV); CLK1 promotes expression of HIV-1 Gag while CLK2 dramatically suppresses synthesis of viral structural proteins [29]. It has also been reported that over-expression of CLKs causes the alteration of 5’ splice site selection of Adenovirus E1A and hyperphosphorylation of 75 kDa SR protein [30]. Co-expression of CLK1 or CLK2 with PTP-1B in HEK 293 cells leads to a twofold stimulation of phosphatase activity by phosphorylation of residue Ser50 in PTP-1B, which is responsible for its enzymatic activity [15]. In cancer and related cells, CLK1 regulates an alternative mRNA splicing factor 45 (SPF45) overexpression through an antagonistic mechanism (SPF45-induced exon 6 exclusion from fas mRNA). CLK1 regulates SPF45 splice site utilization through positive and negative regulations of splicing mechanisms which cause an increase in SPF45 stability, phosphorylation and regulation of mRNA binding (Fig. 3). Inhibition of CLK1 expression leads to SPF45 degradation through a proteasome-dependent pathway. SPF45 overexpression causes enhanced migration and invasion, depending on the...
Fig. (2). Phosphorylation of SRSF1 in nuclear–cytoplasmic localization by SRPK1 and CLK1 [23].

Fig. (3). Action of CLK1 on phosphorylation of SPF45 [27]. Biochemical regulation (identified phosphorylation sites and regulation of fibronectin and cortactin) by CLK1 [31-34].

Fig. (4). Binding mode of (a) 10-Z-Hymenialdisine (pdb: 1Z57) and (b) substituted indole inhibitor KHCB-19 (pdb: 2VAG) on CLK1.
SYNTHETIC INHIBITORS OF CLK1

In comparison to other kinases of CMGC group, relatively fewer efforts have been recorded toward discovery and development of small molecule inhibitors of CLK1. Representative members of small molecule CLK1 inhibitors reported in the literature are shown in (Fig. 6). Logical data also claimed benzothiazole derivatives (structurally analogous to TG003) as potent inhibitors of CLK1 and CLK2 [75]. Another patent from Chronogen, Inc revealed some quinoline derivatives (15) as potent ATP competitive inhibitors of CLK1 [76].

Mott and co-workers discovered a novel class of CLK inhibitors based upon core 6-arylquinazolin-4-amine scaffold [10]. Compound 16 was identified as highly selective and potent inhibitor of CLK1, CLK4, and Dyrk1A with IC50 values 37 nM, 50 nM and 27 nM respectively. Molecular modeling studies suggest that the above compound and related analogues inhibit the CLK isozymes through binding at the ATP binding domain [21,71]. Subsequent reports from the same group recorded systematic modifications on compound 16 which resulted in the development of NCGC00188654 CID: 4496823 (17) and NCGC00010037 (18, ML106) [9]. Both the compounds were found to retain good inhibitory potency against CLK1/CLK4 and selectivity across a panel of kinases.

Recently, Coombs and co-workers reported discovery of some substituted pyrimidines as potent inhibitors of the CLK and Dyrk kinases [77]. They initially screened the NCGC library of 375 pyrimidines having three different core structures against CLK4, which led to the discovery of three novel lead molecules (19-21) each belonging to different chemotype. Each lead molecule was subsequently optimized through an extensive SAR analysis resulting in three novel candidates (22-24) with low-nanomolar inhibitory activity against CLK1, CLK2, CLK4, Dyrk1A and Dyrk1B. Kinome scan analysis of the three most active compounds against 442 kinases showed these inhibitors (22-24) to be highly selective for the CLK and Dyrk
families.

Several molecules based upon the imidazopyridazine scaffold (K00523 (25), K00152 (26), K00135 (27) and K00482 (28)) were reported to possess moderate to significant inhibitory activity on CLK1 [12]. Iodotubericidin (29), an adenosine derivative shows weak CLK1 inhibitory activity, whereas BIM-9 (30), a well-known bisindolyl maleimide-based protein kinase inhibitor exhibited modest CLK1 inhibitory activity [12]. N,N'-bis(5-arylidene-4-oxo-3,5-dihydro-4H-imidazol-2-yl)diamines were found to be a new class of compounds with significant CLK1 inhibitory potency [78]. Two inhibitors of this class 31 (IC_{50} = 0.68 µM) and 32 (IC_{50} = 0.61 µM) inhibited CLK1 at submicro-molar concentrations. Compound 32 also showed micromolar inhibitory potency against other structurally related kinases like GSK a/p (IC_{50} = 2.7 µM), DYRK-1A (IC_{50} = 5.4 µM), and CLK3 (IC_{50} = 1.5 µM) [78].

Loidreau and co-workers described the microwave-assisted synthesis and kinase inhibitory activity (CDK5, GSK3a/p, Dyrk1A, CLK1 and CK1) of benzo[b]thieno[3,2-d]pyrimidin-4-amines (33) and their pyrido (34, 35) and pyrazino derivatives (36) [79]. They found that among the three tricyclic scaffolds studied, N-arylpyrido[30,20:4,5]thieno[3,2-d]pyrimidin-4-amines showed interesting selectivity and inhibitory potency towards CK1 and CLK1 kinases over the other tested kinases. Loidreau et al further continued their exploration of this tricyclic core with N-aryl-7-methoxybenzo[b]furo[3,2-d]pyrimidin-4-amine (37) and N-arylbenzo[b]thieno[3,2-
Lead molecules discovered in a screen against CLK4 using an NCOC library of 373 pyrimidines.

(Fig. 6) contd....
(Fig. 6) contd....
Fig. (6). Synthetic inhibitors of CLK. The important domains of the molecules responsible for the interaction with the active site of the enzymes are highlighted.

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