Protein Kinase

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Protein kinases (PKs) are enzymes that catalyze the transfer of the terminal phosphate group from ATP to a protein acceptor, mainly to serine, threonine, and tyrosine residues.

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1. Introduction

Kinases are a group of enzymes that catalyze the transfer of the terminal γ-phosphate from ATP to the hydroxyl group of an acceptor substrate, thus participating in a huge variety of cellular processes, such as proliferation, apoptosis, metabolism, transcription, or antibiotic resistance, among others. These phosphorylation reactions can be reversed by the corresponding phosphatases. These important discoveries concerning reversible protein phosphorylation as a biological regulatory mechanism were recognized by the award of the Nobel Prize in Physiology or Medicine in 1992 to Edmond H. Fischer and Edwin G. Krebs [1]. Even though all kinases catalyze the same phosphoryl transfer reaction, there is a wide diversity in their structures and substrates [2], which include proteins, lipids, carbohydrates, amino acids, vitamins, and cofactors. According to their structure and sequence, kinases have been classified into 30 families [3], of which the protein kinase (PK) family is the largest comprising one of the most abundant protein families in mammalian genomes [4].

PKs catalyze protein phosphorylation, mainly of serine, threonine, and tyrosine residues, and play a critical role in cellular signaling pathways that affect crucial cell processes, such as growth, differentiation, and metabolism [5]. Phosphorylated proteins can initiate a downstream cascade of reactions, resulting in a vast range of responses including activation or inhibition of enzyme activities [6], changes in biological activity, as well as facilitating or perturbing movement between subcellular compartments, and initiating or interrupting protein–protein interactions [7].

PK activity was first observed in 1954 in an enzyme that catalyzed casein phosphorylation [8]. The first evidence that one PK can activate another was the observation that cAMP-dependent protein kinase A (PKA) activates phosphorylase kinase [9]. The same kinase, PKA, was also described as the first example of enzyme inhibition by phosphorylation, which inhibits glycogen synthase [10]. Since then, characterization of PKs has been widely addressed. In 2002, Manning et al. [11] published the PK complement of the human genome, the so-called kinome, classifying it in 518 members, with most PKs (478) belonging to a single superfamily containing a eukaryotic PK (ePK) catalytic domain, while the other 40 PK genes were reported to belong to a few atypical families (aPK) with proteins showing biochemical kinase activity, but with no sequence similarity to the ePK domain.

2. Protein Kinase Targets in the Control of Virus of the *Flaviviridae* Family

2.1. The AGC Kinase

AGC kinase group is named for the initials of its members, kinases related to cAMP-dependent protein kinase 1 (PKA), cGMP-dependent protein kinase (PKG), and protein kinase C (PKC). The group is formed by serine/threonine protein kinases that share common characteristic structural features, including the presence of a hydrophobic sequence motif close to the C-terminal lobe of the catalytic core [12]. The group comprises more than 60 members classified into 14 subfamilies: PDK1, AKT/PKB, SGK, PKA, PKG, PKC, PKN/PRK, RSK, NDR, MAST, YANK, DMPK, GRK, and SGK494.

Drugs targeting AGC kinases have been shown to be valuable pharmacological candidates for targeting distinct flaviviruses. The PKA inhibitor PKI significantly reduces ZIKV replication by inhibiting the synthesis of viral genomes, producing minimal cytotoxicity on human endothelial cells and astrocytes, highly susceptible to ZIKV infection [13]. The PKG inhibitor Rp-8-pCPT-cGMPS drastically decreases DENV replication in human HEK293T cell culture, while the PKG activator 8-Br-PET-cGMP produces an increase in DENV yield [14]. Similarly, WNV has been reported to upregulate PKCs during infection [91], and the PKC inhibitors calphostin C and chelerythrine have been reported to reduce WNV

multiplication [15]. In contrast, in vitro number of DENV viral copies increased upon treatment with the PKC inhibitor bisindolylmaleimide I, whilst the opposite effect was observed in baby hamster kidney (BHK-21) cells treated with the PKC activator phorbol 12-myristate 13-acetate, thus indicating that inhibition of PKC activity promotes DENV replication [16].

2.2. Calcium Calmodulin Dependent Kinases (CAMK)

The CAMKs are serine/threonine kinases activated by increases in the concentration of intracellular calcium ions (Ca^{2+}). The activity of this group is mainly regulated by the Ca^{2+} receptor protein calmodulin (CaM). They are classified into two different types: substrate-specific and multi-functional CAMKs. The former can phosphorylate only a specific substrate, while the latter can phosphorylate multiple targets.

A broad antiviral activity against members of the *Flaviviridae* family has been shown by drugs targeting CAMKs. For instance, SFV785 has selective effects on MAPKAPK5 kinase activity, and has been reported to inhibit DENV and YFV viral yield by altering the co-localization of the structural E protein with the DENV replication complexes. This effect on MAPKAPK5 kinase activity did not inhibit DENV RNA synthesis or translation. [17]. Similarly, inhibition of CHK2 with CHK2 inhibitor II effectively reduced JEV production in a range of human cell lines, such as A549, HEK293T, U87 and BE(2)C [18]. Fluvastatine, an inhibitor of DCLK1, downregulated HCV replication in GS5 cell culture, derived from human hepatoma Huh 7.5 cell line, without exerting any negative effect on cell viability [98]. In addition, silencing of Pim Kinase with siRNA, or pharmacological inhibition with SGI-1776, inhibits HCV at an early entry step when human hepatoma Huh 6 and human primary hepatocyte cell cultures were infected [19].

On the other hand, activation of proteins belonging to the CAMK group has also been reported as being effective. Activation of AMPK with PF-06409577 impaired viral replication in WNV, ZIKV, and DENV infected Vero (monkey) and BHK-21 (hamster) cell lines [20], and other pharmacological activators of AMPK, such as AICAR, metformin, and GSK621 have been described as attenuating ZIKV replication in endothelial cell culture [21]. Likewise, liraglutide, which activates AMPK in an AMPK/TORC2-dependent pathway, inhibits HCV replication in the human hepatoma Huh 7 cell line [22].

2.3. Casein Kinase 1 (CK1)

CK1 is a monomeric serine-threonine protein kinase with seven isoforms. Pharmacologic inhibition with d4776 was reported to decrease YFV yield in infected human HEK293 cells [23]; however, inhibition of the CK1ε isoform with IC261 promotes WNV infection by suppressing the production of type I interferon, either in vitro, after infection in human HEK293 cells, or using an in vivo model, since CK1ε-deficient mice produced less IFN-β and were more susceptible to WNV infection [24]. On the other hand, the specific CKII inhibitor, 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT), was shown to disrupt virion biogenesis in human hepatoma Huh 7.5 cell infected with HCV [25]. This inhibitor was described as enhancing HCV genotype 1a production in the same cell line [26], thus revealing that genotype-specific differences should be taken into account for potential future pharmacological use of this compound.

2.4. CMGC Kinases

CMGC kinases, such as the AGC group, are named with the initials of family members; cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase (GSK), and CDC-like kinase (CLK). This group consists of 63 family members highly conserved in eukaryotic organisms.

Drugs targeting CMGC kinases have been described as antiviral candidates against several flaviviruses, as well as against HCV. In the case of DENV, different studies have highlighted the MAPK/ERK pathway as essential for replication, since DENV infection can directly activate proteins in this pathway, including JNK, p38, NTRK1, MAPKAPK5, and c-src/FYN kinases [27]. JNK and p38 kinase inhibitors were reported to significantly reduce DENV protein synthesis and viral yield in infected monocyte-derived macrophages obtained from human peripheral blood [28]. The p38 inhibitor SB203580 prevented lymphopenia, hematocrit increase, and inflammation in human PBMCs, THP-1, and KU812 cell lines infected with DENV [29], and improved the survival rate in DENV-infected AG129 mice [30]. In vitro inhibition of ZIKV virion production was also observed with this agent in infected human endothelial cells and astrocytes [13] and with the related SB202190 [31]. Furthermore, ZIKV production in human neural cell lines was hindered upon treatment with structurally unrelated CDK inhibitors, such as seliciclib, PHA-690509 [32], and Cdk1/2 inhibitor III [33], which also suppressed DENV and JEV viral propagation in the human hepatoma Huh 7 cell line. Selective inhibition of the MAPK/ERK pathway has also been described to block infectious HCV production in infected human Huh 7.5 cells [34], and the inhibitor BmKDfsin3, obtained from scorpion (*Mesobuthus martensii*) venom, also decreases HCV replication by downregulation of the p38 MAPK signal pathway in Huh7.5.1 and HEK293T infected cell lines [35]. Finally, an SRPK inhibitor (SRPIN340) suppressed the expression of an HCV subgenomic replicon and the in vitro replication in Huh7 and Huh7.5.1 cell lines of the HCV-JFH1 clone in a dose-dependent manner [36].

2.5. Tyrosine Kinases (TKs)

TK phosphorylates almost exclusively on tyrosine residues, whilst most other kinases are selective for serine or threonine. This group is classified into two subtypes, receptor (RTKs) and non-receptor, or cytoplasmic TKs (CTKs), depending on their function in transmembrane signaling, or within the cell mediating signal transduction to the nucleus, respectively. RTKs have transmembrane and extracellular domains, whilst CTKs do not. RTKs primarily transmit extracellular signals into the cell. CTKs are, generally located within the cytoplasm, although often membrane-associated.

TKs have been deeply studied and their involvement in flavivirus replication has been widely reported [37]. The c-Src/Fyn kinase has been identified as a cellular target in DENV RNA replication. The pharmacological inhibitor saracatinib (AZD0530) inhibits virion assembly of DENV in human Huh7 and HEK293T infected cell lines [38]. Likewise, compound 16i, another Src inhibitor, was reported to suppress DENV replication at low micromolar concentrations with no significant toxicity to the host cell [39], thus validating the Src family of TKs as potential drug targets for the development of treatments against DENV infection. Other SFKs are also implicated in DENV infection; Abl inhibitor GNF-2 interferes with DENV replication in human hepatoma Huh-7 and Vero African green monkey kidney infected cells [80]**.** The involvement of other TKs, such as those acting on the JAK/STAT3 pathway, has been described. JAK2 and JAK3 inhibitors have been reported to reduce DENV-induced phosphorylation of STAT3 and cell migration, as well as production of the chemokines IL-8 and RANTES in infected hepatocytes [40]. Likewise, WNV-infected human SK-N-MC and HEK 293 cells treated with the SFK inhibitor PP2 show a decrease in viral titers, whilst there was no effect on intracellular levels of either viral RNA or protein, thus suggesting that the drug has no effect on the early stages of replication [41]. Two inhibitors of AXL phosphorylation, cabozantinib, and R428, significantly impair ZIKV infection of human endothelial cells [42]. TKs have also been related to hepacivirus replication, and the EGFR inhibitor erlotinib inhibited HCV infection in a dose-dependent manner in different cell lines, such as Huh7, Huh7.5.1 cells and primary human hepatocytes [37].

Additionally, diverse TKs have been described as broad-spectrum anti-viral agents. A covalent host BTK inhibitor, QL-XII-47, was reported to inhibit DENV, WNV, and ZIKV in the human Huh 7 cell line [43]. Furthermore, the kinase inhibitor SFV785 was shown to reduce secretion of infectious DENV and YFV virions in Vero and BHK-21 infected cells [44]. Likewise, inhibition of EGFR kinase activity via induction of IFN-α inducible protein 6 (IFI6), an IFN-stimulated gene (ISG), strongly inhibited DENV either in vitro or in vivo in AG129 mice [45], WNV [46], and HCV infection [47], either in vitro or in vivo, in AG129 mice. The wide spectrum TK inhibitor dasatinib was reported to reduce virion assembly in DENV via Fyn kinase in human Huh7 and HEK293T infected cell lines, and to inhibit HCV infection via EphA2 TK in different cell lines, such as Huh7, Huh7.5.1 cells, and primary human hepatocytes [37].

2.6. Tyrosine Kinase-Like (TKL)

TKL kinases are serine-threonine protein kinases with sequence similarity to TKs, but lacking TK-specific motifs. This is the most recently defined PK group, and families within it are little related to each other. As with TKs, TKL kinases are classified into receptor and non-receptor kinases, and are distributed in eight major families.

The main target among TKL kinases reported as antiviral candidates against flaviviruses are Receptor Interacting Protein Kinases (RIPKs), key mediators of cellular signaling that are essential for the early control of diverse pathogens [48]. Among them, RIPK3 has been described as involved in neuroinflammation and neuronal death during JEV infection, tested either in vitro using neuro2a cells or in vivo, in wild type and RIPK3–/– mice [49]. RIPK3 signaling also restricted viral replication in ZIKV [50] and WNV [51] infections in mice.

2.7. Other PKs

There are several families included in the ePKs identified by Manning [11] that lack sequence similarity with the previously described ePK groups, and, thus, they are catalogued in a separate group.

Numerous drugs targeting this heterogeneous group have shown antiviral activity against flaviviruses and HCV infections. For instance, the IRE1 kinase inhibitor KIRA6 reduces viral RNA levels in ZIKV infected the human HeLa cell line [52]. DENV infection has been widely reported to be inhibited by inhibitors of different members of the group, as exemplified by treatment with pyruvate kinase PKM2 inhibitor in DENV-infected U937 cells [84], the AurKB inhibitor ZM 447439 in DENV-infected Huh-7 cells [53], and the NAK family inhibitors, sunitinib and erlotinib (AAK1 and GAK subfamilies inhibitors respectively) in DENV-infected Huh-7 cells [54]. PKR is modulated by cyclophilin A, triggering antiviral responses to inhibit HCV infection in Huh 7 cell line [55], and the PKR2 inhibitor HA1077, also known as fasudil restricted HCV replication in mice [56]. NAK inhibitors also affect HCV assembly, which was disrupted by treatments with erlotinib, dasatinib, or isothiazolo[5,4-b]pyridine (GAK subfamily inhibitors) in Huh 7.5 cells [57], and sunitinib or PKC-412 (AAK1 subfamily inhibitors) tested in Huh7.5 and 293T cell lines [58][59]. Erlotinib and dasatinib are not NAK specific, and inhibit TKs, as

mentioned above, although the authors of these studies pointed to GAK inhibition as the cause of HCV inhibition [106]. BX795, a TBK1/IKKε inhibitor, showed effects against HCV infection in the Huh 7 cells [60]. As a consequence of its involvement in autopprotein kinases; phosphorylation; antivirals; flaviviruses; hepatitis C virushagy, endoplasmic reticulum (ER) stress, and unfolded protein response (UPR), PERK has been associated with apoptosis in JEV infection either in vitro in neuro2a and BHK-21 cells or in vivo in BALB/c mice [61], DENV infected canine MDCK cells [62][63], and WNV infected SK-N-MC human neuroblastoma cells [64].

Pharmacological modulation of the STE, RGC, and atypical kinases has yet to be linked to flaviviral infection.

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