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A historical overview of protein kinase PKR: from its discovery and mechanism of action to its clinical and therapeutic implications

Title in Spanish: *Una visión histórica de la proteína quinasa PKR: desde su descubrimiento y mecanismo de acción hasta sus implicaciones clínicas y terapéuticas*

María Ángel García Chaves [1]

[1]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, Instituto de Salud Carlos III, 28029 Madrid, Spain.

ABSTRACT: The protein kinase R (PKR, also called EIF2AK2) is an interferon-inducible double-stranded RNA protein kinase with multiple effects on cells. PKR plays an active part in the cellular response to numerous types of stress, with a critical role in the host's interferon-induced antiviral defence mechanisms. PKR has been extensively studied and documented for its relevance as a cell growth regulator, and more recently analysed in connection with metabolism, inflammatory processes, cancer, and neurodegenerative diseases. The present review will summarise, in chronological order, the state of the knowledge about this kinase as well as the contributions we have done at the National Centre of Biotechnology regarding the regulation and mechanisms of action of PKR. Specific mention will be made of the studies that the author leads at the University Hospital Complex of Granada, showing the importance that PKR has as a target of both conventional chemotherapeutics and novel drugs, and its potential as a biomarker and therapeutic target in various pathologies.

RESUMEN: PKR, también llamada EIF2AK, es una proteína quinasa de respuesta a ARN de doble cadena inducida por interferón que participa en múltiples efectos en las células. PKR contribuye de forma activa en la respuesta celular a numerosos tipos de estrés, teniendo una importante función en el mecanismo de defensa antiviral del hospedador inducido por los interferones. PKR ha sido estudiada intensamente a lo largo del tiempo documentando su relevancia también como modulador del crecimiento celular, y más recientemente implicándola en metabolismo, procesos inflamatorios, cáncer y enfermedades neurodegenerativas. En esta revisión se resume de forma cronológica, el conocimiento adquirido sobre esta quinasa y nuestras contribuciones en el mecanismo de acción y regulación de PKR llevadas a cabo en el Centro Nacional de Biotecnología. Además, con especial interés, se describen los estudios que la autora lidera en el Complejo Hospitalario Universitario de Granada mostrando la importancia que PKR tiene como diana molecular de quimioterapéuticos convencionales y nuevos fármacos así como su potencial como biomarcador y diana terapéutica en varias enfermedades.

*Corresponding Author: magciaves@yahoo.com
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1. INTRODUCTION

The Protein kinase R (PKR), also called eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2), is an interferon-inducible double-stranded RNA protein kinase with multiple effects in cells (1, 2). PKR actively contributes to the cellular response to numerous types of stress, with a critical role in the antiviral defence mechanism of the host induced by interferons. Interferon cytokines (IFNs) have a wide range of biological functions, including antiviral, antiproliferative and immunomodulatory properties (3). The cloning of interferon genes, the structure of the ligand and their receptors, and the signalling pathways and transcription of IFN-induced genes have been instrumental in reaching an understanding of how these molecules exert their function in the cell (4, 5). Among the molecules with important biological function induced by IFN is PKR, which was discovered after the important observations carried out by the Metz and Kerr groups in the National Institute for Medical Research, London. In August 1972, an article by Dr Metz and Dr Esteban published in the *Nature* journal showed how viral RNA could be detected in cells pretreated with interferon and infected with vaccinia virus, and how protein synthesis ceased early after infection. They concluded that the effect of interferon must therefore be at the stage of translation rather than transcription (6). A few months later, Dr Kerr's group described the inhibition of viral encephalomyocarditis messenger ribonucleic acid translation in L-cell extracts (7). Subsequently, they showed the effect that the synthetic form of dsRNA (poly dIdC) had on translation (8). These seminal studies led to the identification in 1976 of a protein with dsRNA-dependent kinase activity involved in translation modulation (9). The kinase activity responsible

for inhibition was then under active search in different laboratories after the demonstration that a dsRNA-dependent kinase could inhibit translation through phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) in rabbit reticulocyte lysates (10). In addition to eIF2α, the kinase activity was found to phosphorylate a p68 protein in human cells and a p65 protein in murine cells (11). The generation of polyclonal and monoclonal antibodies against the human p68 protein allowed determining that this was the kinase itself (12). To achieve its cloning, which was performed at the Pasteur Institute (13), this kinase first had to be purified by immunoaffinity with specific monoclonal antibodies (14). Then, the purified protein was injected into mice in presence of poly(A).poly(U) (15). Different names were given to this kinase, such as p68 protein kinase, DAI (Double stranded Activated Inhibitor), dsRNA-dependent protein kinase, and P1/eIF2α kinase, until the decision was made to give PKR a consensus name: Protein Kinase dsRNA-dependent (16). The human PKR gene consists of 17 exons (whereas the mouse gene has 16) and is encoded from a single gene located on human chromosome 2p21-22 and on mouse chromosome 17E2 (17). PKR, which is expressed constitutively in mammalian cells, has an IFN-stimulated response element (ISRE) in its determined promoter, required for transcriptional induction by type I IFN, but also a kinase conserved sequence (KCS) motif with an important role in basal transcription in absence of cytokine tratment (18).

Since these findings were made, PKR has been extensively studied to document its relevance as a first-line defence mechanism against infection and as regulator of cell growth, and more recently it has also been analysed for its role in metabolism, inflammatory processes, and age-related diseases. In fact, numerous pieces of original research and reviews regarding PKR action have been published over the years. My research about the mechanism of action of this kinase started in close collaboration with Dr Gil and co-workers in the group led by Dr Esteban at the National Centre for Biotechnology (from 2000 until 2008), and has provided key insights into the consequences that PKR activation has at the cellular level but also at the clinical and therapeutic levels. For this reason our group was invited to review the knowledge of PKR in 2006-2007 (1, 2). These reviews are being referenced by other studies looking into the mechanism of action and the clinical implications of this kinase in several pathologies. In fact, the reviews we published under the direction of Dr Esteban, with my contribution as the first author, in the journals *Microbiology and Molecular Biology Reviews* and *Biochimie* have been cited over 600 times to this date (Scopus database).

The present review will perform a chronological account of our major contributions to the knowledge of the mechanisms of action and regulation of PKR, as well as the decisive contributions of several international groups. Specific mention will be made of the studies that the author leads at the University Hospital Complex of Granada (Instituto de Investigación Biosanitaria de Granada, ibs.GRANADA) since 2009, in the group currently directed by Dr Marchal, which show the importance that PKR has as a target of conventional chemotherapeutics and novel drugs. In addition, special consideration will be given to future studies necessary to validate its use as a biomarker and a therapeutic target in various pathologies.

2. MECHANISME OF ACTION OF PKR

PKR is a serine-threonine kinase composed of a kinase domain, shared by the other eukaryotic initiation factor 2α (eIF2 α) kinases: general control nonrepressed 2 serine/threonine-protein kinase (GCN2), heme-regulated serine/threonine-protein kinase (HRI), and PKR-like endoplasmic reticulum kinase (PERK) (19). In addition, PKR has two dsRNA binding domains that constitute the regulatory domain (20). Although the main direct PKR activator is dsRNA (produced during infection by several viruses and detected at low doses in mammalian cells), PKR is also activated by a variety of forms of cellular stress described throughout the manuscript. PKR, in response to specific stress signals, is activated by autophosphorylation and leads to the phosphorylation of eIF-2α impairing its activity, which results in the inhibition of protein synthesis and the induction of apoptosis (1, 2). In addition to its translational regulatory function, PKR has a role in signal transduction and transcriptional control through the inhibitor of κB (IkB)/nuclear factor κB (NF-κB) pathways. Furthermore, PKR is involved in various pathways that activate and engage a number of transcription factors controlling the expression of multiple genes, including interferon regulatory factor 1 (IRF-1), signal transducers and activators of transcription factors (STATs), mitogen-activated protein kinases (MAPKs), and p53, among others (1, 2). All these events indicate that PKR protein needs to be highly regulated. In fact, numerous studies have found PKR to be dysregulated in most cancers, neurodegenerative diseases, and other pathologies.

The main mechanisms of PKR activation by dsRNA, as well as its effects on the inhibition of protein synthesis through the phosphorylation of eIF2α, had already been thoroughly characterised before the start of my research in the field. However, the mechanism by which PKR activation induces the activation of the transcription factor NF-kB was still in the early stages of research when I joined Dr Esteban's group, where we made, along with Dr Gil, Dr Alcami and co-workers, some key contributions. These researchs, along with those of other groups, will be discussed in the following sections

2.1. PKR activation

Although the main direct PKR activator is dsRNA, PKR is also activated by a variety of cellular stresses, including cytokine, calcium stress, oxidative stress, endoplasmic reticulum stress, lipo-stress, amyloid-β (Aβ) peptide accumulation, polyanions such as heparin, and several drugs among others (1, 2, 21, 22), or through the

PKR associated activator (PACT) (23, 24). PKR is also an intermediary in TLR signalling (25). PKR is engaged in dsRNA-activated TLR3 signalling, recruited by a TAK1-containing complex in response to dsRNA binding to the TLR3 receptor. In addition, PKR integrates and transmits these signals not only to eIF-2α and the translational machinery, but also to various factors such as STAT IRF1, p53, JNK, and p38, as well as engages the NF-kB pathway (1, 2, 26). In non-stressed cells, PKR is in a monomeric latent state due to the autoinhibitory effect of its dsRBD, which occludes the KD and regulates the activation of the kinase. The different dsRNA molecules are recognised and bound by PKR through the two N-terminal dsRBM, resulting in PKR activation and autophosphorylation (27). The structure of the PKR dsRNA binding domain was determined by nuclear magnetic resonance (28) offering a satisfactory explanation for the length required of dsRNA molecules to be effective PKR activators. Most natural dsRNA activators of PKR are synthesised in virus-infected cells as by-products of viral replication or transcription. For RNA viruses, dsRNA replicative forms are obligatory intermediates for the synthesis of new genomic RNA copies. Complex DNA viruses such as vaccinia virus (VV), adenovirus, or herpes simplex virus (HSV) have open reading frames in opposite orientation; they produce overlapping mRNA transcripts that can fold to form dsRNA stretches responsible for PKR activation in infected cells (1, 2). After binding dsRNA, PKR undergoes a number of conformational changes that relieve the autoinhibitory interactions of the enzyme and allow subsequent substrate recognition. Biochemical and genetic data have underscored the importance of homodimerisation in PKR activation (29). After homodimerisation, PKR undergoes rapid autophosphorylation in a stretch of amino acids termed the activation segment. Among others, residues Thr446 and Thr451 in this segment are consistently phosphorylated during activation (29, 30). This further stabilises PKR dimerisation, which in turn increases the catalytic activity of the kinase. Whether of viral origin or pIC, dsRNA thus not only induces effects on translation, but also influences various signal transduction pathways that affect different transcriptional activities. As such, PKR mediates the dsRNA-induced transcription of many genes through engagement of multiple transcription pathways (1, 2, 26).

2.2. Translation regulation by PKR

A number of reports have provided insights into the mechanism of PKR activation and eIF2α phosphorylation, which consists of a three-step pathway in which dimerisation of the kinase domain triggers autophosphorylation, in turn promoting specific recognition of eIF2α. PKR activation-segment phosphorylation on Thr446 promotes substrate recognition and phosphorylation, although it has been reported that phosphorylation at tyrosine residues in PKR also contributes to the binding to dsRNA, autophosphorylation, and eIF2α phosphorylation (31). To this day, a total of 14 phosphorylation sites have been identified in PKR, but only 12 are biochemically verified, and 8 have been functionally characterised (32). Although phosphorylation of most sites serves to augment kinase activity toward eIF2α, only phosphorylation of T451 is required to generate an active kinase (32). As predicted for a translation regulator, PKR is associated to ribosomes, mainly to 40S subunits (33). Ribosomal association of PKR appears to be mediated by the dsRBD, strengthening the role of these domains in the correct regulation of PKR activity. PKR localisation in ribosomes offers a satisfactory explanation for its local activation in response to limited stimulus, as reported by several studies (1, 2, 33). Two models have recently suggested new evidence for a sentinel model of ribotoxin-induced PKR activation (34). One possibility is a sentinel model in which PKR monomers basally associate with the ribosome and rRNA. Upon interaction with a ribotoxin, one or more portions of rRNA reposition and thereby promote dimerisation of the PKR monomers followed by autophosphorylation and self-activation. A second possibility is a sequential mode whereby a ribotoxin first associates with rRNA and inflicts damage and/or alters its structure, thereby exposing new double-stranded (ds)rRNA regions. This could sequentially elicit the binding of two or more PKR monomers in close proximity to the damaged site, followed by the dimerisation of these monomers and finally the autophosphorylation and self-activation of the kinase (34).

2.3. NF-κB activation by PKR: identification of TRAF family proteins linking PKR with NF-κB activation

The NF-κB family of transcription factors controls the expression of genes involved in immune and inflammatory responses, cell differentiation, and apoptosis, among others (35). NF-κB activation is primarily regulated through its interaction with the family of inhibitory proteins IκB which retain NF-κB in the cytoplasm. Phosphorylation of IκB on two conserved serine residues is mediated by the IκB kinase complex (IKK complex) in response to a variety of stimuli, leading to its subsequent ubiquitin-dependent degradation by the 26S proteasome. This allows NF-κB translocation to the nucleus, where it can activate the transcription of a number of genes including those encoding cytokines, chemokines, cell surface receptors, and adhesion molecules (36). The IKK complex contains a structural protein termed IKKγ or NEMO and two kinase subunits, IKKα and IKKβ (37). The first clues suggesting a role for PKR in NF-κB activation arose from observations in 1989 that dsRNA could induce NF-κB activity in different cell lines (38). Subsequent experiments using the kinase inhibitor 2-aminopurine suggested a role for PKR in this process. Additional evidence came from the analysis of NF-κB activation following dsRNA treatment in cells lacking PKR expression. When PKR expression was downregulated using 2-5A antisense oligonucleotides, diminished NF-κB activation was observed in response to dsRNA, with no significant change in the response to TNF-α (39). The design of mice deficient in PKR expression in 1995 allowed carry out many critical experiments for studying the mechanism of action of the

kinase (40). In fact, experiments performed with PKR$^{-/-}$ MEF showed that NF-κB activation was impaired in response to pIC treatment. As a consequence of the NF-κB activation impairment, PKR$^{-/-}$ MEF show defects in IFN production compared with wt MEF (40). The first experiments to show the ability of PKR to activate NF-κB in the context of viral infection came from the laboratory of Dr Esteban in 1999-2000. These experiments were performed with human cells and vaccinia virus recombinants expressing PKR in an inducible manner (Figure 1), showing that IκBα phosphorylation on serines 32 and 36 precedes its degradation and translocation of NF-κB to the nucleus (41, 42).

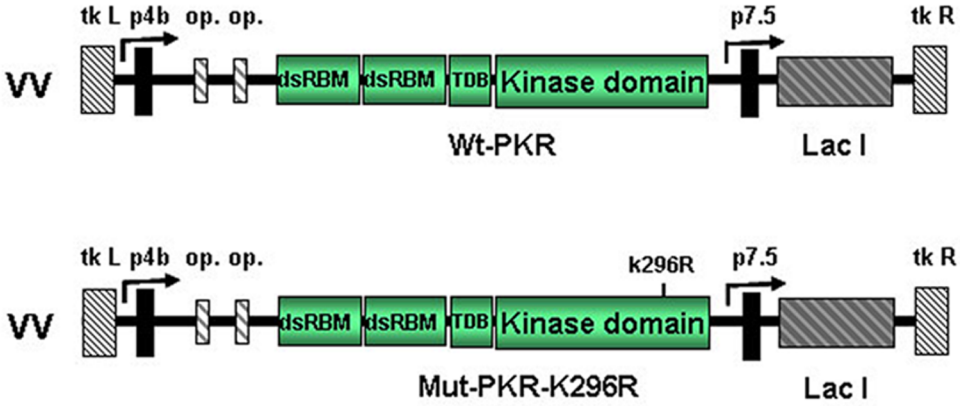


Figure 1. Scheme of the recombinant vaccinia virus vectors. The Western Reserve strain of vaccinia virus was used to generate recombinant viruses expressing the human wild type (WT) and the mutant PKR proteins. The recombinant genes were inserted in the TK locus of viral genome. PKR expression is regulated by the lac I repressor gene that is under the control of VV early-late promoter p7.5. The mutant PKR, K296R has lost the catalityc activity of PKR due to substitution of the lysine 296 by arginine. These vectors allow over-express proteins in culture cell lines under IPTG (isopropil-β-D-1-tiogalactopiranósido) induction. Most of our interest genes were over-expressed using these recombinant vectors.

Initial reports indicated that PKR was the protein kinase that phosphorylated IκBα directly in response to dsRNA, based on *in vivo* and *in vitro* evidence (43). Although PKR appears to be necessary for transducing this signal (1, 2, 43, 44), later evidence pointed to an indirect role for PKR in IκB phosphorylation. Mutant cells lacking IKKγ were unable to induce NF-κB in response to pIC treatment (45). Although the kinase NIK was initially proposed to be downstream of PKR in the IKK activation process, the participation of NIK in this pathway seems dubious by virtue of current knowledge of IKK signalling (1, 2, 37). Although the evidence is consistent with PKR playing a role in the activation of IKK in response to dsRNA, the nature of such role is still unclear. In fact, research groups have disagreed on whether the catalytic activity of PKR was necessary or not for the successful activation of NF-kB. In this regard, the studies led by Dr Esteban's group made significant contributions and provided some interesting experiments demonstrating the essential action of the catalytic domain of PKR. In this exciting and pioneering environment is where I began my research of the mechanism of action of PKR as a PhD student under the direction of Dr Esteban. Our experiments showed that the association with the IKK complex seems to involve the PKR catalytic domain, as mutational analysis suggested (46). Experiments using PKR$^{-/-}$ cells suggested that PKR catalytic activity is needed for IKK activation using vaccinia virus recombinants expressing several PKR mutants (Figure 1), (46). Similar results were found by Dr Williams's group, in a study in which the complementation of PKR$^{-/-}$ MEF with plasmid expressing wt PKR (but not with a catalytically inactive mutant) restored appropriate NF-κB and IRF-1 activation (44). However, experiments from other groups carried out on NIH 3T3 cells suggested that a catalytically inactive PKR mutant is a poor IKK activator, but when PKR is expressed at high levels it can activate IKK efficiently (47). Purified PKR, either wt or mutant K296R, activated the recombinant IKK protein, suggesting that PKR catalytic activity was not needed in the process (48, 49). It has been suggested that the variety of models used in the different groups can yield either result, also accepting that the catalytic activity of PKR may or may not be necessary to activate NF-κB depending on the type of stimulus triggering the process, and the cellular stress stage.

A major part of my doctoral thesis looked into ways to decipher the mechanism by which PKR activates NF-κB transcription factor. Sharing first authorship with Dr Gil, in 2004 we published an original article in *Molecular and Cellular Biology* about how TRAF family proteins link PKR with NF-κB activation. Since then, this piece of research has been cited over 80 times. Several pathway-specific adapter proteins, such as members of the TRAF (TNF receptor associated factors) family, MyD88, TIRAP, and TRIF, act as mediators that link different pathways with IKK activation (1, 2, 50). TRAF proteins have emerged as key signal transducers not only downstream of TNF receptors, but also in other pathways (50). We identified two putative TRAF-interacting motifs in the PKR sequence, and the viability of the PKR/ TRAF

interaction was suggested by bioinformatic analysis and confirmed *in vivo* (51). The interaction between PKR and TRAF2 or TRAF5 was shown to be dependent on PKR dimerisation and is functionally relevant, as we demonstrated in cells genetically deficient in TRAF2 and TRAF5 or after expression of TRAF dominant negative molecules, suggesting that TRAF family proteins act downstream of PKR and signal towards the activation of NF-κB (51). With our study as a starting point, other groups have linked PKR/TRAF with several signalling pathways with significant relevance in the induction and activation of Interferon type I, such as TLR-related pathways (52). Recently PKR/TRAFs have been also involved in the protection against a disease caused by a non-viral pathogen (53).

3.4. PKR involvement in different signalling pathways: New PKR networks through microarray analysis

The number of functions that PKR plays is in correlation with its participation in numerous signalling pathways that have been described over several studies (1, 2, 26) (Figure 2).

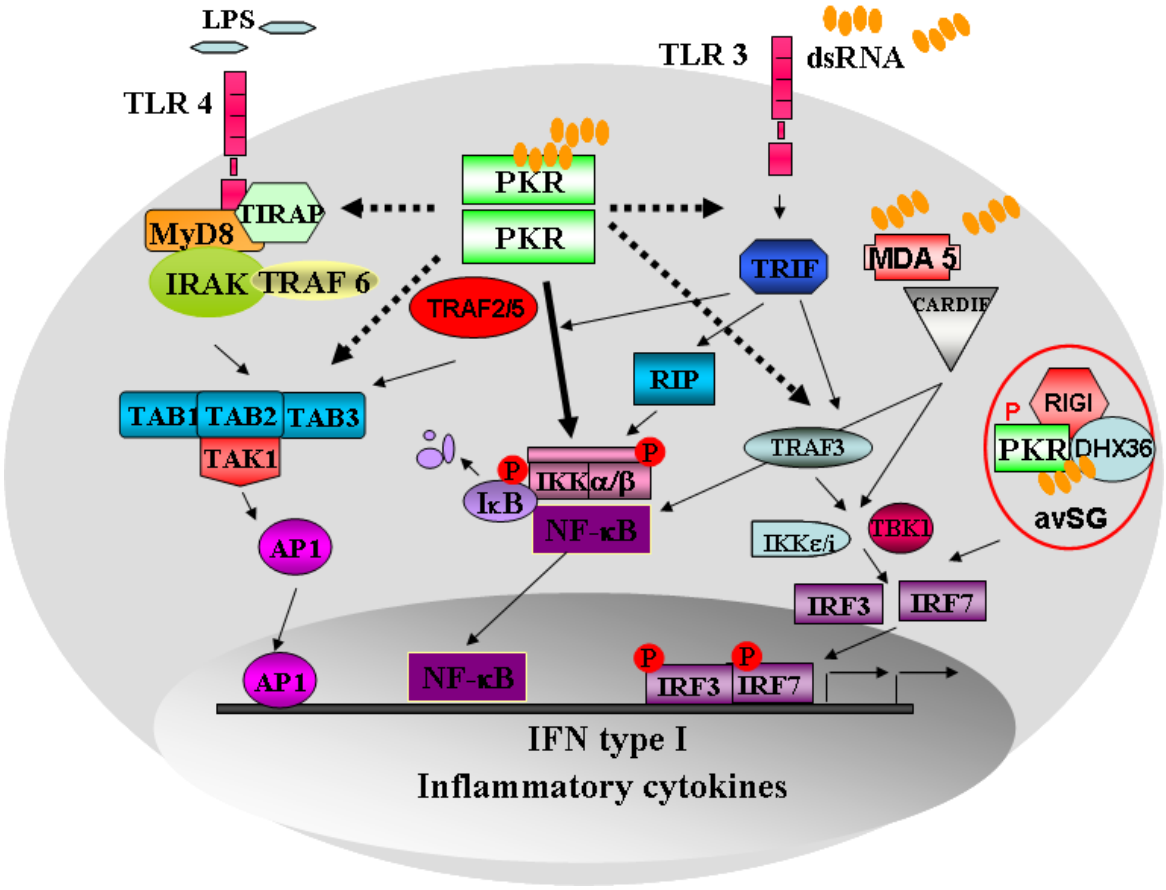


Figure 2. PKR is a cellular sensor implicated in the detection of viral RNA and subsequent interferon gene expression. Viral RNA sensors as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), and the membrane sensors as the Tool-like receptors TLRs were discovered after PKR, adding complexity to viral host recognition. PKR is an intermediary component in TLR signalling. PKR is implicated in the LPS/TLR4-mediated pathway probably recruited by the TIRAP complex. PKR is also involved during the dsRNA/TLR3 pathway, recruited by a TAK1-containing complex. Moreover, PKR is crucial for the IFN- α/β production in response to MDA5-dependent viruses. In addition PKR link RIG-I in the antiviral stress granules function and formation.

PKR was one of the first cellular sensors implicated in the detection of viral RNA and subsequent interferon (IFN) gene expression. However, this clear picture of PKR antiviral function was complicated by the discovery of cytosolic viral RNA sensors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), and membrane sensors as the toll-like receptors TLRs (Figure 2), (54). Since then, the role of PKR as RNA sensor was minimised, and the focus moved to the role of other sensors. However, it has been demonstrated that PKR plays an essential role in IFN-α/β production in response to viral infections by regulating the integrity of IFN-β transcripts. By using PKR-deficient cells, it has been possible to show that PKR is crucial for IFN- α/β production in response to MDA5-dependent viruses like encephalomyocarditis virus (EMCV), Theiler's murine encephalomyelitis virus (TMEV) and Semliki Forest virus (SFV), but not to RIG-I-dependent viruses such as Sendai virus or influenza (55). However, it has been suggested recently a new link between PKR and RIG-I in the antiviral stress granules function and formation (56, 57). In addition, the idea of PKR as key

regulator of IFN protein synthesis has been also supported by other studies demonstrating that PKR plays a non-redundant role in the IFN response to viral infections (54).

On the other hand, it is well-known that PKR is a potent activator of NF-kB, thereby inducing IFN transcription in concerted action with interferon regulatory factors 3 and 7 (IRF-3/7). PKR has been also implicated in the activation of interferon regulatory factor 1 (IRF 1), whose expression is strongly upregulated upon viral infection, and which acts mainly as a transcriptional activator of IFN-α/ β gene expression (5, 44, 54). PKR also controls IFN and dsRNA signalling pathways by modulation of STAT1 and STAT3 transcription factors. PKR-knockout cells are defective in STAT1 phosphorylation on Ser727, resulting in a 4-fold decrease in STAT1 dependent transactivation (58). STAT1 is also a target for PKR-mediated activation in response to lipopolysaccharide (LPS) in glial cells (59). PKR also associates to STAT3, and is required for full STAT3 activation in response to platelet-derived growth factor (PDGF). As proposed for STAT1, PKR regulates the Erk activation ultimately involved in STAT3 phosphorylation (60). On the other hand, since it also regulates STATs transcription factors, PKR has recently been described to be involved in the differentiation of chondrocytes through the modulation of STAT1 and Sox-9 expression (61).

In addition to its well-established role in the interferon response, PKR is involved in many cellular pathways exerting various functions on cell growth and tumourigenesis (1, 2, 26). For example, the link between PKR and p53 has been described fundamentally in cancer cells where there is a bidirectional and complex regulatory relationship between both proteins. PKR interacts directly with the C-terminal part of p53 and phosphorylates p53 at the Ser392 residue (62). In addition, PKR is able to promote the proteasomal degradation of p53 in association with GSK-3β and mouse double minute 2 homologue (Mdm2), independently of translational control (57). Moreover, it has been demonstrated that the ability of p53 to cause cell cycle arrest and regulate transcription of target genes was impaired in PKR-knockout cells (62). In fact, it has been suggested that PKR is a p53 target gene that plays an important role in the tumour-suppressing function of p53 (63).

Moreover, PKR is an activator for signalling cascades involving stress-activated protein kinases, and is described to mediate Jun kinase (JNK) and mitogen-activated protein kinase p38 (MAPK) activation in response to specific stimuli (1, 26). For full activation in response to LPS or cytokines such as IFN-γ, interleukin (IL)-1, or tumour necrosis factor (TNF)-α, both p38 and JNK are dependent on PKR (64). Moreover, PKR interacts with and activates mitogen-activated protein kinase kinase 6 (MKK6) in response to double-stranded RNA stimulation (65).

In order to improve the state of knowledge about new host genes affected by PKR, we used human cDNA microarrays to identify, in infected cells, genes differentially expressed after PKR expression, and analysed the requirements of catalytic activity of the enzyme (66). To express PKR, we used vaccinia virus recombinants producing wild type PKR and the catalytically inactive mutant K296R (Figure 1). Most regulated genes were classified according to biological function, including apoptosis, stress, defence, and immune response. A total of 111 genes were regulated specifically by PKR catalytic activity, highlighting the upregulation of the ATF-3 transcription factor, involved in stress-induced β-cell apoptosis. Using null cells for ATF-3 and for the p65 subunit of NF-κB, we showed that induction of apoptosis by PKR at late times of infection was dependent on ATF-3 expression and regulated by NF-κB activation. The host genes affected by PKR, identified using human cDNA microarrays, together the ATF3 implication were published by Dr Guerra and co-workers in 2005 under the direction of Dr Esteban in the *Journal of Biological Chemistry* (66).

2.5. PKR is a potent pro-apoptotic protein: caspases activation by intrinsic and extrinsic routes

Cell death by apoptosis is a genetic program of multicellular organisms which implements the ordered removal of damaged or unwanted cells during development and in adult life. Deregulation of the apoptotic process can lead to pathological conditions such as cancer, autoimmunity, and neurodegeneration (67). Induction of apoptosis is a common response to viral infection. Although it may represent an antiviral mechanism that acts by rapidly eliminating infected cells and preventing viral spread, virus-induced apoptosis can also have important pathological implications. Moreover, apoptosis cell death is an important event during some chemotherapy treatments in cancer diseases.

The first evidence that PKR was involved in apoptosis was suggested by Dr Esteban's group in 1994 using HeLa cells infected with a VV recombinant vector that expressed the enzyme under inducible conditions (Figure 1), (68). The role of PKR in apoptosis was reinforced by studies developed by other groups with 3T3 cells expressing a noncatalytic mutant PKR or using MEF derived from $PKR^{-/-}$ mice (69, 70). Since then, it has been clearly demonstrated that PKR mediates the apoptosis induced by several viruses such us poxviruses, influenza, EMCV, VSV, etc., probably through dsRNA production (1, 2). PKR also regulates apoptosis induced in the absence of viral infection. PKR was shown to mediate the apoptosis observed during Alzheimer's disease (71) and induced by oncogenes such as IRF1 or E2F-1, or triggered in response to dsRNA, TNFα, LPS, tunicamycin, serum starvation, or IL-3 withdrawal (1, 2). Many of the stimuli that trigger PKR-dependent apoptosis in the absence of viral infection rely on PACT/RAX activation. PACT/RAX mediates PKR activation and subsequent apoptosis in response not only to cytokines and serum withdrawal, but also to chemotherapy, ethanol, and viral infection (23, 24, 72). Analysis of the role of PKR effectors in mediating cell death suggests an intricate pathway. To distinct degrees, at least eIF-2α, NF-kB, ATF-3, and p53 have been implicated

in mediating PKR-induced apoptosis (1, 2, 26). Our research has provided interesting results involving all these factors. Initial evidence that PKR phosphorylation of eIF-2α is involved in apoptosis induction came from studies showing that PKR-mediated apoptosis can be inhibited by expressing an eIF-2α dominant-negative mutant (73). Using the VV expression system (Figure 1), the group of Dr Esteban showed that apoptosis induced by PKR expression was prevented by coexpression of an eIF-2α 51A mutant (73). Moreover, they described the involvement of NF-κB activation in the apoptosis induced by PKR using proteasome inhibitors that block IκBα degradation, or by coexpressing dominant negative forms of IκBα. These observations may appear paradoxical, as NF-κB is often classified as a prosurvival factor that prevents apoptosis. However, there is considerable evidence for the context of NF-κB as a pro- or antiapoptotic factor, depending on the stimulus that triggers the apoptosis (1, 2, 41, 42). Subsequently, we contributed to the discovery of a new transcription factor involved in the apoptosis induced by PKR (66). The ATF-3 transcription factor, involved in stress-induced β-cell apoptosis, was upregulated after PKR over-expression. Activation of endogenous PKR with a VV mutant lacking the viral protein E3L, a PKR inhibitor, triggered an increase in ATF-3 expression that was not observed in PKR-knockout cells. Using null cells for ATF-3 and for the p65 subunit of NF-κB, we showed that induction of apoptosis by PKR at late stages of infection was dependent on ATF-3 expression and regulated by NF-κB activation (66). Other potential mediators of PKR-induced apoptosis are the components of the Arf/p53 pathway. The p53 pathway is a critical regulator of apoptosis (1, 2), and the link between PKR and this pathway has been established at several levels. Our recently published work in the field is discussed below. IRF-1 is another PKR target involved in apoptosis induction (44).

To understand how the activation of PKR effectors regulates apoptosis induction, it is necessary to understand how the apoptotic machinery integrates these signals. Proteolytic enzymes such as caspases are important effector molecules in apoptosis. Caspases are synthesised as inactive proforms and, upon activation, they cleave next to aspartate residues. Activation of caspases can be initiated from different entry points, as for example at the plasma membrane upon ligation of death receptor (receptor pathway/extrinsic pathway) or at the mitochondria (mitochondrial pathway/intrinsic pathway) level. Stimulation of death receptors of the tumour necrosis factor (TNF) receptor superfamily, such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors, results in activation of the initiator caspase-8, which can propagate the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3. The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, among others, from the mitochondrial intermembrane space. The release of cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex. Links between the receptor and the mitochondrial pathway exist at different levels. Upon death receptor triggering, activation of caspase-8 may result in cleavage of Bid and Bax, Bcl-2 family proteins which in turn translocate to mitochondria to release cytochrome c, thereby initiating a mitochondrial amplification loop. Finally, activation of caspase-3 triggers the induction of cell death by apoptosis (1, 74), (Figure 3).

Our research on the mechanism of action involved in apoptosis induced by PKR has been very active. The group led by Dr Esteban showed that PKR-induced apoptosis involves mainly the FADD/caspase 8 pathway. Expression of a FADD dominant-negative mutant or MC159L (from molluscum contagiosum virus, MCV) by using VV recombinants blocked PKR-induced apoptosis and decreased caspase 8 activity, showing that PKR triggers apoptosis through FADDmediated activation of caspase 8 (75). In 2002, we found that PKR expression by VV recombinants also induces caspase 9 activation, which correlates with Bax protein translocation to the mitochondria and cytochrome c release to the cytoplasm, resulting in mitochondrion depolarisation (76). The PKR-induced caspase 9 biochemical process occurred downstream of caspase 8 activation, as treating cells with an inhibitor of caspase 9 results in partial prevention of PKR-induced apoptosis (76). Also a part of my doctoral thesis, this work showed both the extrinsic and intrinsic pathways that PKR induces during apoptosis (Figure 3).

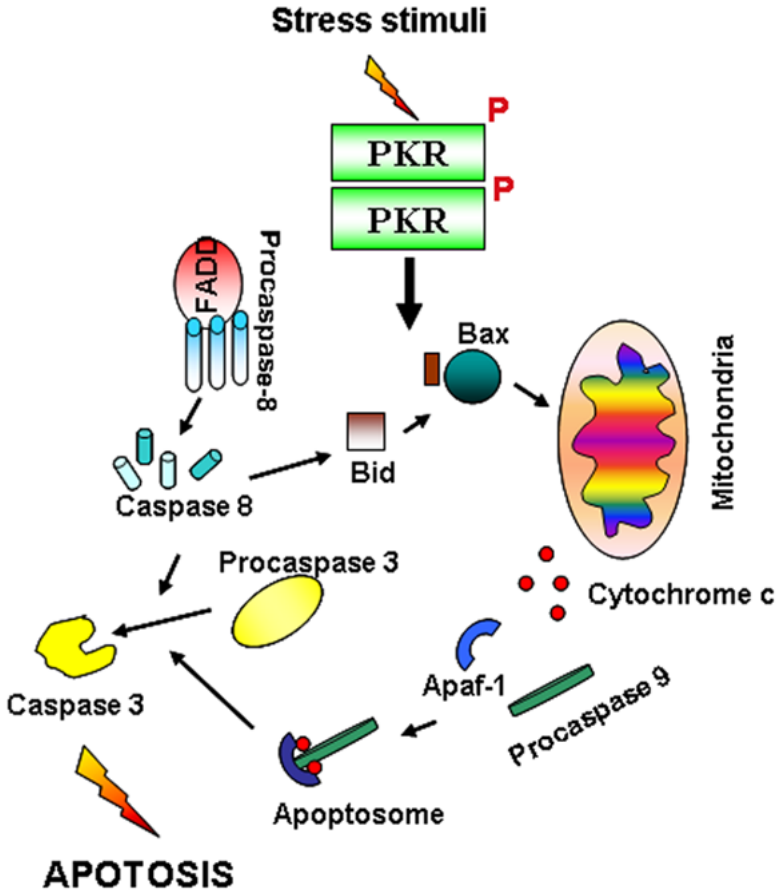


Figure 3. PKR induces apoptosis activating both, the extrinsic and the intrinsic routes of caspases activation. Through interaction with FADD, PKR activates caspase-8, which in turn activates the conversion of Procaspase-3 and provokes Bid/Bax interaction, release of cytochrome c from the mitochondria and formation of the apoptosome (Apaf-1/cytochrome c/caspase-9). Both pathways result in activation of caspase-3 and degradation of DNA, thus resulting in programmed cell death or apoptosis.

3. PKR REGULATION

The importance of PKR function in antiviral defence, cell growth, differentiation, stress response, and immune modulation is further highlighted by the existence of specific direct and indirect modulators.

3.1. Viral modulators of PKR effect: E3L from vaccinia virus, LANA 2 from Kaposi's sarcoma herpesvirus, and polyprotein from hepatitis c virus as subjects of our interest

Since the IFN-induced cellular antiviral response is the primary defence mechanism against virus infections, many viruses have developed a means to counteract the induction or effects of IFN (1, 2). Viruses use a number of strategies to counteract dsRNA-dependent pathways, and specifically to avoid the deleterious effects of the PKR and other IFN-induced systems. Numerous viral proteins have been identified as able to avoid the PKR effect, directly binding PKR or indirectly preventing eIF2α phosphorylation. One recently-researched mechanism that contributes indirectly by inhibiting the effect of PKR is mediated by growth arrest and DNA damage-inducible protein 34 (GADD34), which physically interacts with phosphatase-1 cofactor PP1c leading to enhanced dephosphorylation of eIF-2α (77). In addition, some viral mRNA could initiate translation in an eIF2-independent manner by means of a dedicated RNA structure that stalls the scanning 40S ribosome on the initiation codon (78). Viral inhibitors are normally expressed from the onset of infection to maintain PKR inactive until the virus cycle is completed. Elimination of PKR inhibitors from these viruses generally has a severe impact on virus replication and pathogenesis. Viruses disarmed of PKR inhibitors usually replicate at lower levels than wild-type viruses in normal cultured cells, and show an attenuated phenotype in animals. A detailed analysis of different viral proteins inhibiting PKR was made in our reviews in 2006 (1, 2). In the present review, an update with new viral proteins is displayed in Table 1. In addition, our contribution to the study of different viral proteins modulating the PKR effect is also discussed.

Vaccinia virus (VV) and its derivative viruses have been widely used by the group of Dr Esteban, and are still being researched as very promising vaccines against several infections and diseases (HIV, HCV, malaria, cancer, etc.) (79). VV is relatively resistant to the antiviral

effects of IFN and is able to rescue the replication of IFN-sensitive viruses such as VSV and EMCV following co-infection. Earlier findings suggested that VV resistance to IFN was related to interference between the virus and the IFN system (80). Later studies showed a more complex landscape, with the VV genome encoding secreted proteins that bind to receptors and ligands of cytokines and chemokines (1, 2, 81), and at least two proteins, K3L and E3L, with the ability to inhibit intracellular IFN-induced pathways. More recently, another protein (K1) has been identified as a possible PKR inhibitor (82). The K3L protein is expressed early in VV infection. K3L protein binds directly to PKR in vitro, and yeast two-hybrid interaction assays have localised the K3L protein-binding site to the C-terminal half of the PKR kinase domain. Competition binding experiments and sequence homology between K3L and the N-terminal one-third of eIF-2α (72% similarity and 28% identity) suggest that PKR recognises K3L and eIF-2α by a common mechanism (1, 2). In this way, K3L protein inhibits autophosphorylation of PKR, blocking the subsequent inhibition of protein synthesis (1, 2, 83). The role of the E3L gene as an inhibitor of apoptosis was first detected after infection of HeLa cells with an E3L deletion mutant of VV by Dr Esteban´s group (1, 68). The VV E3L gene encodes two proteins, p25 and p20, expressed early in infection. E3L is a host range gene, necessary for efficient VV replication in several cell lines, and is required for VV pathogenesis. The E3L protein is a dsRNA-binding protein where the carboxy-terminal domain of E3L encodes the conserved motif that binds dsRNA. The N-terminal domain required for neurovirulence is involved in the direct inhibition of PKR activation, nuclear localisation, and Z-DNA binding (1, 2, 84). E3L also inhibits PKR by direct interaction with PKR, leading to heterodimer formation (1, 85). Our contribution in the study of E3L protein showed that E3L expression in NIH 3T3 cells conferred antiapoptotic and oncogenic properties. To analyse E3L effects over cellular metabolism in a virus-free system, we generated stable mouse 3T3 cell lines expressing E3L (86). Expression of E3L resulted in inhibition of eIF-2α phosphorylation and IkBα degradation in response to dsRNA. Antiviral responses induced by IFN-α/β were partially impaired in 3T3-E3L cells, as we determined by a viability assay upon VSV infection. E3L expression also conferred resistance to dsRNA-triggered apoptosis. Interestingly, cells expressing E3L grew faster than control cells, and showed increased expression of cyclin A and decreased levels of p27Kip1. E3L cooperated with H-ras in a focus formation assay, and NIH3T3 E3L cells formed solid tumours when injected in nude mice. Overall, our findings reveal that interference of E3L protein with several cellular pathways results in promotion of cellular growth, impairment of antiviral activity, and resistance to apoptosis (86). These results (which we published with my contribution as the first author in *Oncogen* journal in 2002) together with the previously described works, led me to writing my doctoral thesis "Mechanism of action and regulation of Protein Kinase induced by Interferon: PKR/ Mecanismo de acción y regulación de la Proteina Quinasa inducida por Interferón:PKR", which received the Special Award (Premio Extraordinario) of the Autonomous University of Madrid.

Recently, in collaboration with the group of Dr Rivas, we have described the regulation of the E3L protein by small ubiquitin-like modifier proteins. E3L interacts with SUMO1 through a small ubiquitin-like modifier (SUMO)-interacting motif (SIM). SIM integrity is required for maintaining the stability of the viral protein and for the covalent conjugation of E3 to SUMO1 or SUMO2, a modification that has a negative effect on the E3L transcriptional transactivation of several apoptotic genes (87). My collaboration with Dr Rivas started in 2003, and since then we have made several significant contributions to the knowledge about the link between tumour suppressors and antiviral activity, resulting in some of the works described in the present review.

Kaposi's sarcoma herpesvirus (KSHV). In collaboration with Dr Rivas, we showed that the viral protein LANA2 codified by KSHV inhibits apoptosis and the PKR-mediated translational block (88), and we identified a nuclear export signal with important implications for the function of this viral protein (89).

Hepatitis C virus (HCV). We looked deeper into the mechanisms of action of other viral proteins modulating PKR and, in order to analyse the effects of hepatitis C virus (HCV) on the antiviral response of the host, we developed a novel vaccinia virus (VV)-based delivery system polyprotein expression (VT7-HCV7.9), where structural and nonstructural (except part of NS5B) proteins of HCV ORF from genotype 1b were efficiently expressed and produced, and timely regulated in mammalian cell lines. HCV polyprotein expression caused a severe cytopathological effect in human cells as a result of the inhibition of protein synthesis and apoptosis induction triggered by the activation of the IFN-induced enzymes PKR and RNase L systems (90).

Table 1: Viral inhibitors of PKR effect

Viruses	Inhibitor Proteins	References
Family *Adenoviridae*		
Adenovirus	E1B-55K/E4orf6	(91)
Family *Bunyaviridae*		
Hantavirus	ANDV NP	(92)
Rift valley fiver, RVFV	NSs	(93)
Family *Coronaviridae*		
Infectious bronchitis virus, IBV	Nsp2	(94)
Family *Filoviridae*		
Ebola viros EBOV	Vp35	(95)
Family *Flaviviridae*		
Japanese encephalitis virus, JEV	NS2A	(96)
	NS4A	(97)
Dengue virus, DENV	NS5A	(98)
Hepatitis C Virus, HCV	E2	(99)
Family *Herpesviridae*		
Herpes simplex virus, HSV	US11, γ34.5	(100)
Epstein Barr, EBV	SM	(101)
Kaposi's sarcoma-associated herpesvirus, KHSV	LANA2	(88)
	vIRF-2	(102)
Cytomegalovirus, CMV	TRS1,IRS1	(103)
Family *Ortomixiviridae*	NP	(104)
Influenza A virus	NS1	(105)
Family *Paramixoviridae*		
Respiratory sincitial virus, RSV	RSV NP	(106)
Family Reoviridae		
Reovirus	P17	(107)
Family *Retroviridae*		
Human Immnodeficiency virus	TAT	(108)
Family *Togaviridae*		
Chikungunya	Nsp4	(77,109)
Family *Poxviridae*		
Vaccinia virus, VV	E3L	(68, 84)
	K3L	(83)
	K1	(82)

3.2. PKR modulation by cellular components

Other functions of PKR (apart from its antiviral activity) have surfaced with the discovery of numerous proteins and other cellular structures modulating the kinase. Moreover, some cellular proteins also modulate PKR to regulate the antiviral and immunomodulatory responses.

p58IPK. A member of the tetratricopeptide repeat family, p58IPK is was the first reported cellular inhibitor of PKR (110). p58IPK interacts directly with PKR and inhibits its kinase activity by preventing dimerisation. Influenza virus partially evades the host's antiviral response by recruiting p58IPK to repress PKR-mediated eIF-2α phosphorylation (1, 2, 111). In the absence of viral infection, p58IPK overexpression results in malignant transformation. Although the exact mechanism has not been defined, it has been suggested that p58IPK transforms cells by interfering with PKR-regulated pathways. PKR inhibition by p58IPK can stimulate cell growth by disrupting PKR-dependent control of mRNA translation and by blocking PKR-dependent apoptosis (1, 112). Interestingly, an independent antiviral link has recently been identified between both proteins, PKR and p58IPK, involving joint degeneration in mice (113).

PACT. The first protein described as PKR activator was PACT (PKR activating protein) able to activate PKR in response to several stresses (1, 23, 24, 72). PACT is a ubiquitously expressed protein that belongs to the family of dsRNA-binding proteins and has three dsRNA-binding domains. Although most studies show that PACT is necessary for PKR activation in response to different toxic compounds, such as arsenic, H2O2, and tunicamycin, PACT-knockout cells and mice did not exhibit significant differences in the response to stressful stimuli compared to a wild-type phenotype (114). Recently, a possible role of PACT as an inhibitor of PKR during HIV-1 replication has been described (115).

TRBP. Trans-activation response RNA-binding protein (TRBP) is a potent PKR modulator that, in turn, regulates PACT protein. TRPB has generated great interest because of its role in RNA interference and microRNA (miRNA) processing (116). Since TRBP inhibits PACT and PKR activation, both modulators are under intensive analysis as potential therapeutic targets. TRBP is a cellular RNA-binding protein isolated by its ability to bind human immunodeficiency virus type 1 (HIV-1) TAR RNA (117, 118). Proposed TRBP functions include inhibition of PKR activation, regulation of cell proliferation, PKR-independent translational activation, modulation of HIV-1 gene expression through its association with TAR, and the control of mRNA translation. TRBP and PKR form a complex using direct protein-protein interaction through their dsRBDs that prevents PKR activation (116).

Chaperones. Chaperones are PKR modulators that have a spectrum of inhibitors currently being tested in preclinical studies with promising expectations (119). PKR is negatively regulated by the heat-shock protein 90 (Hsp90). The drug geldanamycin, an inhibitor of Hsp90, disrupts the interaction of the PKR-Hsp90-p23 complex, allowing PKR activation (1, 2). It has been suggested that Hsp90 contributes to chemotherapy resistance, and its inhibition has potential therapeutic interest. Similar to Hsp90, Hsp70 binds to PKR, inhibits PKR phosphorylation, and prevents apoptosis (120). In stressed cells, Hsp70 binds to the Fanconi anemia complementation group C (FANCC) protein and forms a ternary complex with PKR (1, 2). Consequently, hematopoietic cells with FANCC mutations or downregulated Hsp70 show constitutive PKR activation and sensitivity to various cell stress signals as well as to IFN-based therapy (121). Overall, the evaluation of HSPs in cells with different basal levels of PKR activation may open an interesting field of study on the regulation of PKR and its therapeutic modulation.

NPM. Nucleophosmin (NPM; also known as B23) is an abundant and ubiquitously expressed nucleolar phosphoprotein implicated in ribosome biogenesis (1, 122). It binds nucleic acids, has intrinsic RNase activity and also acts as a molecular chaperone shuttling between the nucleus and cytoplasm. NPM has also been implicated in the acute response to environmental stress and controls cell proliferation (1, 2). NPM is frequently overexpressed in tumours of diverse origin (123), and it is translocated in lymphomas and leukemias. NPM interacts with PKR, inhibiting eIF-2α phosphorylation and PKR-mediated apoptosis (124). It was suggested that the capacity of NPM to inhibit PKR activation could explain how NPM promotes cell proliferation and suppresses the apoptosis pathway (1). We have identified a novel function for NPM involving PKR activity with important antitumour and antiviral consequences, as we analyse in the next section.

3.3. PKR regulation by miRNAs

The first miRNA identified acting on PKR is the noncoding RNA (pre-miR-886) called nc886 (125, 126). MiRNAs, small noncoding regulatory factors 18–25 nt in length, could affect gene expression leading to translational and transcriptional regulation of numerous genes and consequently affect protein expression including kinases. MiRNAs have crucial roles in diverse biological processes, including apoptosis and cell growth. Multiple studies have reported altered miRNA levels in stressed cells or in various disease states, including cancer and neurodegenerative pathologies. Because of its stability and easy detection in body fluids, they are being explored as important biomarkers in various diseases. The extent of miRNA involvement in PKR regulation and activity is still not fully understood, but studies have begun to identify miRNA-mRNA targets of kinases involved in different pathologies. The pre-microRNA nc886 suppresses PKR via direct physical interaction, whereas artificial suppression of nc886 in cholangiocyte cells activated the canonical PKR/eIF2α cell death pathway (126). The importance of the detection of nc886 has been recently revealed in two studies with oncologic patients where nc886 levels in the tumour were related with the progression of the disease (127). Methylation studies

conducted in PBMCs from acute myeloid leukemia and healthy patients have shown epigenetic variability of nc886 (128), suggesting that the expression levels of this regulator will be variable, and may be decisive in diseases where PKR activity is deregulated.

3.4. PKR regulation by compartmental localisation and post-translational modifications: identification of a novel PKR modification by Sumoylation

Although PKR has been identified in nuclear and cytoplasmic fractions, most activities attributed to PKR occur in the cytoplasm; hence, the role of nuclear PKR remains unclear. However, recent studies have attributed clinical and pathological significance to nuclear PKR, mainly in neurodegeneratives diseases (21, 129). Moreover, we and others have shown that PKR can be sequestered in the nucleus by nucleophosmin (1, 2). In acute leukemia, it has been found that PKR exists in diverse molecular weight forms in the nucleus, suggesting that this variance in protein weight is the result of post-translational modifications (129). In fact, PKR can be regulated by different post-translational modifications, including phosphorylation, ubiquitination, and ISGylation. Recent observations resulting from the cooperation between our group and that of Dr Rivas reveal a post-translational modification of PKR by SUMOylation, with a direct role in PKR activation and control of virus infection (130). These results indicate that PKR is modified by both SUMO1 and SUMO2, *in vitro* and *in vivo*. We identified lysine residues Lys-60, Lys-150, and Lys-440 as SUMOylation sites in PKR. Moreover, these results show that SUMO is required for efficient PKR-dsRNA binding, PKR dimerisation, and eIF2α phosphorylation. SUMO potentiated the inhibition of protein synthesis induced by PKR in response to dsRNA, whereas a PKR SUMOylation mutant was impaired in its ability to inhibit protein synthesis and showed reduced capability to control vesicular stomatitis virus replication and to induce apoptosis in response to vesicular stomatitis virus infection (130). Hence, the analysis of the post-translational regulation and compartmentalisation of PKR offer interesting possibilities to control their role in various diseases.

4. PKR INVOLVEMENT IN SEVERAL DISEASES: ITS POTENTIAL AS A BIOMARKER AND THERAPEUTIC TARGET

The study of the mechanism of action and regulation of PKR has opened the door to a new understanding of the implications and therapeutic possibilities of this protein in several diseases as we have recently published in *FASEB Journal* in the review titled "The impact of PKR activation: from neurodegeneration to cancer" under my leadership as corresponding and last author (131). Although further analysis are required involving patient samples, PKR translational research, carried out only in recent years, shows great potential for this kinase as a biomarker and therapeutic target.

My incorporation at the University Hospital Complex of Granada as a Researcher of the National Health System (Miguel Servet Program) was in 2009, with the aim of transferring our knowledge of PKR and Interferon to certain diseases, conducting studies with patients in order to define their clinical utility. Today, I lead a line of research based on the study of Interferon and PKR in several diseases at the Institute of Biomedical Research of Granada (ibs.GRANADA)/University Hospital Complex of Granada, in the group directed by Dr Marchal, collaborating also in the discovery of novel antitumour drugs.

4.1. The role of PKR in Cancer

In addition to its well-established role in the interferon response, PKR is involved in many cellular pathways exerting various functions on cell growth and tumourigenesis (1, 2, 131). However, PKR's exact role in cancer biology remains controversial. Initially, PKR was thought to be a tumour suppressor. The first evidence that PKR controls cell growth, and consequently may function as an inhibitor of cell proliferation, was obtained after overexpression of PKR in mammalian, insect, and yeast cells, where PKR was observed to suppress cell growth (132, 133). Conversely, the expression of several PKR dominant-negative mutants leads to malignant transformation of NIH 3T3 cells, and is able to cause tumourigenesis in nude mice (134, 135). The apoptotic role of PKR was also in agreement with the notion that this protein could be a tumour suppressor. PKR is able to activate the intrinsic and extrinsic apoptotic routes in cancer cells in response to several stimuli, including antitumour drugs (22, 76). Moreover, PKR has been suggested as an essential part of the antitumour activity of tumour suppressors, such as p53 and PTEN (62, 63, 136). The link between PKR and p53 has been described fundamentally in cancer cells where there is a bidirectional and complex regulatory relationship between both proteins. PKR interacts directly with the C-terminal part of p53 and phosphorylates p53 at the Ser392 residue (1, 2, 62). In addition, PKR is able to promote the proteasomal degradation of p53 in association with GSK-3βand mouse double minute 2 homologue (Mdm2), independently of translational control (131). Moreover, it has been demonstrated that the ability of p53 to cause cell cycle arrest and regulate transcription of target genes was impaired in PKR-knockout cells (1, 2). In fact, it has been suggested that PKR is a p53 target gene that plays an important role in the tumour-suppressor function of p53 in response to DNA damage stress. In addition, PKR could be transcriptionally regulated by p53 activity in response to some genotoxic stresses (63). On the other hand, we have evaluated the antitumour activity of overexpressed PKR using VV recombinant (Figure 1) with interesting oncolytic results in mice xenotransplanted with prostate cancer cells, publishing these data as first author in 2010 in *Anales de la Real Academia Nacional de Farmacia* (137). PKR overexppresion was able to reduce tumour volume with the advantage of minimising the specific antibodies induced by the animal against the viral vector in

comparison with the control empty vector, suggesting the potential of VV-PKR construction as an oncolytic vector for cancer (137). A possible mechanism of evasion of PKR activation has been suggested by some cancer cells that are unable to induce apoptosis despite eIF2α phosphorylation status (131). Moreover, it has been reported that PKR is suppressed or inactivated in some malignancies, and increased PKR expression has been shown to correlate with better prognosis in head and neck cancer, melanoma, lung, and colon cancer (1, 131, 138, 139). Furthermore, some evidence shows that PKR may act as a tumour suppressor in leukemia and could play an important role in haematological disorders (129, 131). Other evidence supporting the antitumour role of PKR comes from studies demonstrating the importance of PKR/eIF2α status in cancer response to chemotherapy. Chemotherapeutic drugs such as 5-Fluorouracil, doxorubicin and etoposide are able to induce and activate PKR protein, triggering apoptosis (22, 63, 131, 140).

However, evidence also suggests an antagonist role of PKR in cancer that has challenged the proposed function of PKR as a tumour suppressor. It is now well established that activation of PKR leads to the induction of pro-survival as well as pro-death pathways whose balance depends on the intensity and nature of the activating stimulus as well as the activation or level of expression of the PKR´s modulators (Figure 4).

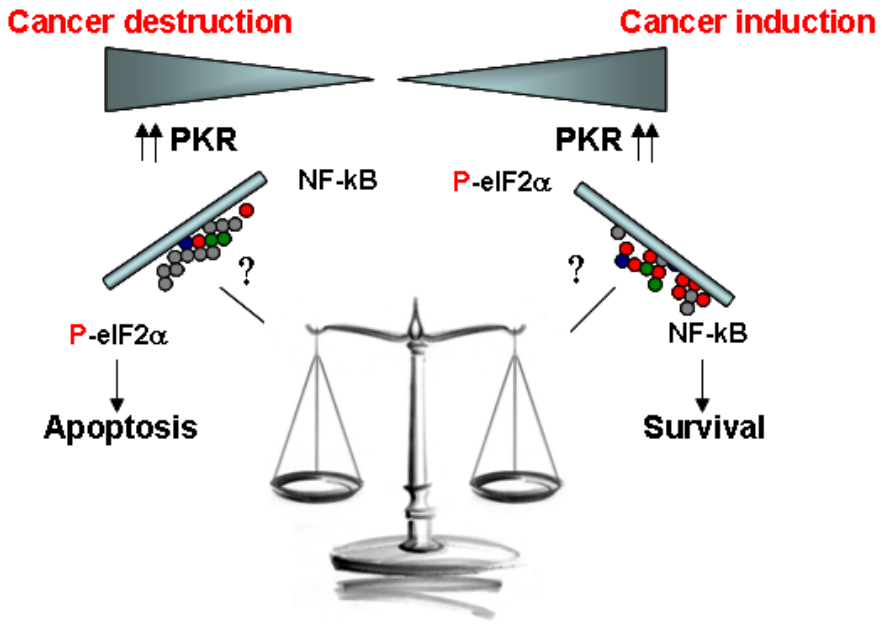


Figure 4. PKR plays an antagonist role in cancer. PKR activation can induce both pro-survival and pro-death pathways. The nature and the intensity of activation stimuli as well as the level of expression of PKR´s modulators could decant the balance of consequences of PKR activation. Understanding the cellular factors and signals that regulate PKR in the different diseases would be extremely valuable from a clinical point of view.

In fact, it has been reported that PKR was overexpressed and linked with malignancy in thyroid carcinoma, broncheoalveolar carcinoma, colon, melanoma, lung, breast, liver cancers and some haematological disorders (131, 141, 142). Moreover, PKR involvement has been suggested in the neoplastic process of the proliferative transcription factor NF-kB (143). Curiously, the finding of different expression patterns of PKR/eIF2α/NF-kB activity, even in the same type of cancer, points to the complexity of the role of PKR in cancer (126). It is important to note that some clinical studies related to PKR analysis did not follow a standardised protocol, differing in the number of tumour samples analysed and in the inclusion criteria of patients, which makes it difficult to compare results between groups. This fact, together with the complexity of PKR signalling and its regulation, highlights the need for consensus on a standardised protocol, specifying patient inclusion criteria, the appropriate number of samples, and the methodology employed.

4.1.a. PKR involvement in the antiviral activity of several tumour suppressors: p53, Arf, and Rb

An increasing number of tumour suppressor genes are induced by interferons and may play an important role in the control of cell proliferation induced by this cytokine. In addition, pathways triggered by both tumour suppressors and IFN converge as common targets for non-related tumour viruses. The inhibition of the IFN response by animal viruses is explained by the fundamental role that IFN plays to control virus infection. However, the reasons why many viruses, including those that do not require the replication of the host, target tumour suppressor pathways are varied and remain under investigation. In fact, oncogenic viruses frequently target the pathways controlled by tumour suppressor genes, suggesting an extra function for these proteins as antiviral factors.

The classical tumour suppressor induced by IFN is p53,

a protein classified as tumour suppressor by its capacity to induce cell cycle arrest and apoptosis in response to a variety of cellular stresses like DNA damage, transcription inhibition, depletion of nucleotide pools, oncogene expression, and heat shock, among others (144). The induction of p53 by IFN was showed by Takaoka et al. in 2003, and the antiviral activity of this tumour suppressor was reinforced by the fact that it is frequently targeted by viral proteins (145). We demonstrated in an elegant way that VSV is impaired as a result of apoptosis induction via p53 activation, using a mice model expressing an extra-copy of p53 called "super p53 mice" (146). Although in this model we did not directly address the role of PKR in the antiviral effect of p53, the close link between both proteins described above suggests great potential for PKR as a mediator, which should be the subject of other, more specific studies.

The alternative reading frame (ARF) is one of the two unrelated products encoded by the INK4a–ARF locus, one of the most frequently mutated genes in cancer. Initially, ARF activity was linked to p53 stabilisation after oncogenic stress, but p53-independent functions have been also described, placing this tumour suppressor as sensor of different types of stress (147). The control exerted by the tumour suppressor Arf on cellular proliferation is crucial to restrict tumour development. Several reports described the activation of ARF after the expression of viral proteins, type I IFN treatment, or after virus infection, suggestive of a physiological role for ARF during virus infection (1, 2). However, due to the well-known connection between ARF and p53, a direct antiviral activity for ARF independently of p53 activation had not been considered before. Our results revealed that ARF can be induced by viral infection and that the expression of ARF reduces viral infectivity. In fact, ARF is protective against IFN-sensitive viruses such as VSV, Sindbis virus, or a recombinant VV rendered IFN sensitive by deletion of the PKR inhibitory gene, E3L. We have shown that this antiviral effect depends in part, on PKR activation mediated by its release from inhibitory complexes with NPM (148). These results provided a new link between tumour suppression and antiviral host defence, an important step to understand the tumourigenic activity of viruses and a crucial learning for the forthcoming use of viruses as therapeutic agents. Under the direction of Dr Rivas, and in collaboration with Dr Esteban and Dr Serrano, these results were published with my contribution as the first author in the prestigious *EMBO Journal* in 2006 (148).

In addition, we suggested other links between tumour suppression and antiviral host defence, with PKR playing a role in the activation of the NF-kB pathway through viral infection. The retinoblastoma protein Rb is a tumour suppressor involved in cell cycle control, differentiation, and inhibition of oncogenic properties (149). We showed that virus replication was increased by the absence of Rb tumour suppressor gene expression, and that Rb was required for the activation of the NF-kB pathway in response to virus infection (150). An analysis of PKR activation in Rb knockout and wild-type MEFs in response to VSV infection revealed decreased levels of both phospho-PKR and phosphor-eI2Fα proteins in the knockout cells, suggesting PKR as a possible mediator. These results revealed a novel role for tumour suppressor Rb in viral infection surveillance and further extend the concept of a link between tumour suppressors and antiviral activity. These results were published in 2009, under the direction of Dr Rivas and Dr Esteban, with my contribution as first author in *Plos One* journal (150).

In summary, these data highlight an important role of different tumour suppressors in the complex innate antiviral host defence, due in part to the involvement of PKR activity. While the field of tumour suppressors with antiviral function is in its infancy, future work will unravel a wider significance of tumour suppressors in host cell defence against pathogens. Understanding how tumour suppressors exert their antiviral function will be relevant in the potential use of viruses as oncolytic agents and for gene therapy in cancer, as we have reviewed in depth in both *Carcinogenesis* and *Future Virology* journals (151, 152).

4.1.b. PKR as a molecular target of the 5-fluorouracil chemotherapeutic drug

The chemotherapeutic drug 5-FU is widely used in the treatment of a range of cancers, being the first and second line of treatment in combination in colorectal cancer patients, and the third line in the palliative care of numerous cancer types. However, adverse effects and resistance to the drug remain major clinical problems. Since defects in the mediators of apoptosis may account for chemoresistance, the identification of new targets involved in 5-FU-induced apoptosis is of great clinical interest. The p53 tumour suppressor has been reported as an important protein involved in 5-FU-induced apoptosis (153). However, several works have shown that apoptosis can also occur in mutant p53 cell lines by a mechanism still unknown, with p53 playing the role of a biomarker of response to 5-FU in tumours pending to find new targets involved in 5-FU sensibility or resistance. We have identified PKR as a key molecular target of 5-FU involved in apoptosis induction in human colon and breast cancer cell lines (154). We analysed PKR distribution and activation, apoptosis induction, and cytotoxic effects during 5-FU and 5-FU/IFNα treatment in several colon and breast cancer cell lines with different p53 status. PKR protein was activated by 5-FU treatment in a p53-independent manner, inducing phosphorylation of eIF-2α and cell death by apoptosis. Furthermore, PKR interference promoted a decreased response to 5-FU treatment and those cells were not affected by the synergistic antitumour activity of 5-FU/IFNα combination. We have shown that PKR is activated in absence of p53 expression and, whereas PKR knockdown decreased 5-FU-mediated apoptosis, cell death was completely abolished in absence of both PKR and p53 proteins. These results suggest the importance of both proteins in 5-FU-induced apoptosis, and the relevance acquired by PKR in

tumour cells where p53 is mutated, considering that more than 50% of colon tumours are deficient in p53 activity. These results, taken together, provide evidence that PKR is a key molecular target of 5-FU with potential relevance in the clinical use of this drug (Figure 5), (154). A method of obtaining useful data to assess and predict the response to treatment with pyrimidine analogues has been protected by international patent (155). My contribution in this field was as corresponding author of published data in *Plos One* journal in 2011 and the main inventor of the patent. Moreover, we have recently published a patent review of 5-FU derivates from 2012 to 2014 and their implications for standard and novel therapies in *Expert Opinion on Therapeutic Patents* journal (157). I am currently the principal investigator of two projects funded by the Andalusian regional government and the Carlos III Institute of Health (ISCIII) whose main objective is to analyse the biomarker potential of PKR in patients with colon cancer treated with therapies based on 5-FU drugs.

4.1.c. Novel antitumour drugs with high capacity to induce eIF2α phosphorylation and cell death by apoptosis: Bozepinib, a promising drug against cancer stem cells, induces PKR-mediated apoptosis and synergises with IFN

Dr Campos, Dr Marchal, Dr Aranega and co-workers have a long history designing and researching new antitumour drugs that are more effective and less toxic than currently standard cancer chemotherapy. My contribution to the group actually directed by Dr Marchal has helped determine the effectiveness of various drugs to induce the phosphorylation of eIF2α and/or PKR-mediated apoptosis. Moreover, my experience with IFN has contributed to create a new line of research in the group that aims to enhance the effectiveness of these novel drugs in combination with biological therapies like interferons, among others.

The most studied drug by our group is called Bozepinib [(RS)-2,6-dichloro-9-[1-(p-nitrobenzenesulfonyl)-1,2,3,5-tetrahydro-4,1- benzoxazepin-3-yl]-9H-purine], which was designed by the group directed by Dr Campos at the University of Granada (Figure 6), (157). Bozepinib is a potent antitumour compound that is able to induce apoptosis in breast cancer cells, as we published in 2011, showing a 50% inhibitory concentration (IC50) of 0.166 μM against the MDA-MB-231 human breast adenocarcinoma cell line and inducing high levels of apoptosis in tumour cells without acute toxicity in mice (157). My contribution in 2013 as corresponding author and last author of the data published in the *Drug Design, Development and Therapy* journal showed that Bozepinib also has antitumour activity in colon cancer cells, showing inhibitory concentration (IC50) values 50% lower than those described for breast cancer cells, and suggesting great potential for this synthetic drug in the treatment of cancer (158). We identified PKR as a target of Bozepinib, being upregulated and activated by the drug. However, p53 was not affected and was not necessary for the induction of apoptosis in either breast or colon cancer cells. In addition, the efficacy of Bozepinib was improved when combined with IFNα cytokine, which enhanced Bozepinib-induced apoptosis with involvement of protein kinase PKR (Figure 5). Moreover, we reported for the first time that, in combined therapy, IFNα induces a clear process of autophagosome formation. Finally, we observed that a minor population of caspase 3-deficient MCF-7 breast cancer cells persisted during long-term treatment with lower doses of Bozepinib and the Bozepinib/IFNα combination. Curiously, this population showed β-galactosidase activity and a percentage of cells arrested in S phase, suggesting that tumour cells enter in senescence, more evidently so in cells treated with the Bozepinib/IFNα combination than in cells treated with Bozepinib or IFNα alone. Considering the resistance of some cancer cells to conventional chemotherapy, these data suggested that combinations enhancing the diversity of the cell death outcome might succeed in delivering more effective and less toxic chemotherapy (158).

Given the great potential exhibited by Bozepinib, we have recently looked into its mechanism of action with very interesting results (159). Bozepinib shows selectivity on cancer cells and an inhibitory effect over kinases involved in carcinogenesis, proliferation, and angiogenesis. The cytotoxic effects of Bozepinib were observed in both breast and colon cancer cells expressing different receptor patterns. Bozepinib inhibited HER-2 (human epidermal growth factor receptor 2) signalling pathway and JNK (c-Jun-N terminal kinase) and ERKs (extracellular signal regulated kinases) kinases. In addition, Bozepinib has an inhibitory effect on AKT (protein kinase B) and VEGF (vascular endothelial growth factor) together with anti-angiogenic and anti-migratory activities. Interestingly, Bozepinib inhibited both mammo- and colonospheres formation regulating genes related to stem properties such as c-MYC, β-CATENIN, SOX2 and GLI-3 hedgehog-signalling repressor ,suggesting activity against cancer stem-like cells (Figure 5). Finally, Bozepinib shows *in vivo* anti-tumour and anti-metastatic efficacy in xenotransplanted nude mice without presenting sub-acute toxicity. These findings support the need for further studies on the therapeutic potential of Bozepinib for cancer patients and were recently published in the *Oncotarget* journal under my leadership as corresponding author (159).

Also designed by the group lead by Dr Campos, other purines-derived compounds with antitumour efficacy that we have characterised as potent inducers of eIF2α phosphorylation and apoptosis include the (R,S)-Benzofused 1,5-Oxatiepine moiety tethered to purines compounds, the (RS)-9-(2,3-dihydro-1,4-benzoxaheteroin-2- ylmethyl)-9H-purines agents, and several enantiospecific heterocycles linked to purines (160-162).

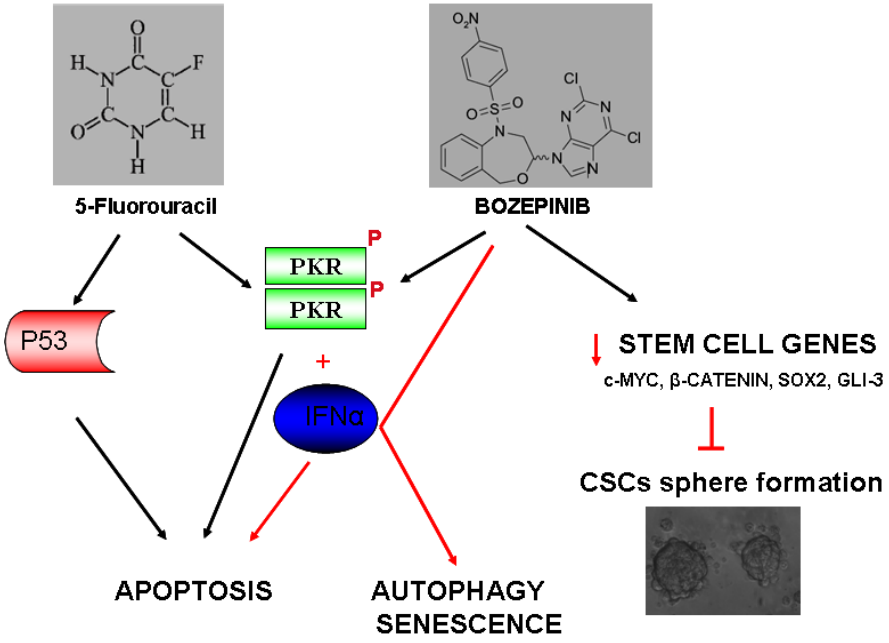
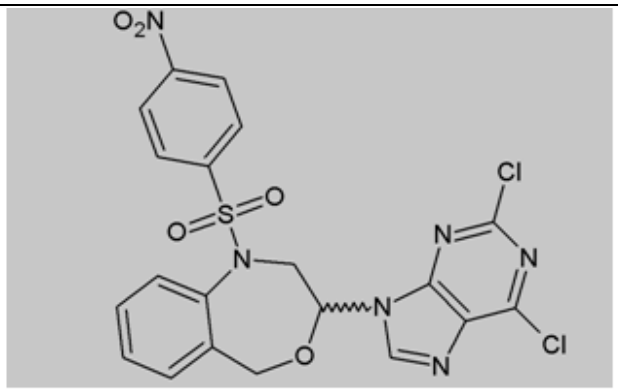


Figure 5. PKR is a molecular target of both anticancer drugs, the chemotherapeutic 5-Fluorouracil and the novel compound Bozepinib. 5-FU and Bozepinib activates PKR inducing cancer cell death by apoptosis. Whereas 5-FU induces PKR activation in a p53-independent manner, Bozepinib does not activate p53. Both drugs synergized its antitumour effect in combination with IFNα.. Moreover Bozepinib is able to induce atophagy and senescense in cancer cells in combination with IFNα. In addition Bozepinib inhibits both mamo and colono-spheres formation regulating genes related to stem properties such as c-MYC, β-CATENIN, SOX2 and GLI-3 hedgehog-signaling repressor.



Bozepinib

[(*RS*)-2,6-dichloro-9-[1-(*p*-nitrobenzenesulfonyl)-1,2,3,5-tetrahydro-4,1- benzoxazepin-3-yl]-9*H*-purine]

Figure 6. Bozepinib structure [(RS)-2,6-dichloro-9-[1-(p-nitrobenzenesulfonyl)-1,2,3,5-tetrahydro-4,1- benzoxazepin-3-yl]-9H-purine]. We have previously published Bozepinib structure in Lopez-Cara *et al.* (157).

4.2. PKR as a molecular target in neurodegenerative diseases

Alzheimer's disease (AD) is a neurodegenerative disorder marked by senile plaques composed of amyloid-β (Aβ) peptide, neurofibrillary tangles made of hyperphosphorylated T-tau protein, neuronal loss, and neuroinflammation, where the apoptotic death characterises most of affected neurons. In 2002, histological methods showed that activated PKR was

accumulated in degenerating neurons in the brain of patients with AD (163). Moreover, a number of recent studies have implicated PKR in the pathogenesis of other neurodegenerative diseases, such as Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (164-166). Previously, it had been demonstrated that the Aβ peptide could induce PKR activation in primary neuronal cultures, triggering cell death by apoptosis (131). Therefore, PKR activation was considered as a secondary consequence of extracellular senile plaque formation contributing to neuronal degeneration. However, recent evidence indicates a direct involvement of PKR activation in the pathology of the disease promoting BACE I expression, an enzyme involved in the accumulation of Aβ peptide (131, 167). Moreover, PKR is also involved in the mechanism of senile plaque formation through tau phosphorylation via glycogen synthase kinase 3β (GSK-3β) activation (168). On the other hand, PKR seems to be involved in the inflammatory process that has been suggested to contribute to AD (131, 169). Several works have shown how PKR inhibition prevented the neuronal loss by apoptosis, suggesting the high potential of PKR as a therapeutic target in neurodegenerative diseases (131, 169). In addition, data showing PKR involvement in learning and memory suggest that PKR inhibition could benefit humans, especially those experiencing age-related memory loss or the most devastating memory loss associated with AD (170). Although different chemical compounds and specific peptides protect neurons from apoptosis by inhibiting PKR, unfortunately they also display high toxicity, induce undesirable effects outside the nervous system, or are not able to cross the blood brain barrier (131, 169). It is therefore necessary to continue looking for new specific inhibitors of PKR. Due to the fact that PKR is an ubiquitous and multifunctional protein, the knowledge of the components involved in how and why PKR is more active in these pathologies would help find alternative drugs to target the abnormal activation of PKR and contribute to the therapeutic control of neurodegenerative diseases.

4.3. Is PKR involved in inflammatory diseases?

Since PKR is induced by IFNα and the proinflammatory NF-kB transcription factor is a target of the kinase, its involvement in inflammatory processes has been suggested in several works (1, 2). In fact, experiments using primary mouse cocultures containing neurons, astrocytes, and microglia have shown that inhibition of PKR prevents activation of NF-kB, as well as a strong decrease in production and release of tumour necrosis factor-α (TNF-α) and interleukin-1b (IL-1b), (171). Several studies have shown a significant increase in various inflammatory mediators in plasma and peripheral blood mononuclear cells of patients with AD compared to age-matched controls (172). Interestingly, levels of both total PKR and phospho-PKR were modified in blood lymphocytes of patients with AD compared with control individuals, and the upregulation of these proteins in cerebrospinal fluid was suggested as a potential biomarker for AD (131, 169).

Moreover, NFkB regulates the cytokines involved in several inflammatory diseases like rheumatoid arthritis and lupus erythematosus, such as IL-6, TNF-α, and IL-1. The kinase PKR has been involved in the induction of TNF-α and IL-6 in response to lipopolysaccharide (LPS) in fibroblasts and alveolar macrophages (173). Interestingly, transcription of IL-1β, TNF-α and IL-6 decreased in mice deficient in the expression of PKR when subjected to a high-fat diet (174). The inhibition of PKR with the C16 compound in PBMCs isolated from patients significantly decreased the expression and production of cytokines IL-1β, TNF-α, and IL-6, suggesting that the inhibition of PKR at the peripheral level can decrease the inflammatory process in Alzheimer's patients (172). However, the effect of PKR inhibition over these cytokines in autoimmune diseases has not yet been explored.

Recently, it has been shown that PKR is an important key element of the inflammasome complex in macrophages. PKR interacts with the NOD-like receptor family, which, together with caspase 1 (Casp-1), integrates this interesting complex, inducing high levels of the HMGB1 cytokine (175). High levels of this cytokine and of IL -1β have also been linked with inflammatory processes involved in AD, autoimmune diseases, and cancer (131). In addition, the PKR gene deletion, or specific drug inhibition, severely diminished inflammasome activation in response to various stimuli (131). Therefore, the analysis of PKR in cancer, neurodegeneration, and autoimmune diseases in the context of the inflammasome complex can provide new evidence on the connection of PKR with these pathologies with an interesting therapeutic potential.

5. CONCLUDING REMARKS AND FUTURE PROSPECTS

Since PKR was discovered, its mechanism of action has been gradually brought to light in several international publications, an effort to which we have made important contributions. The involvement of PKR in interferon-mediated activities, in the induction of apoptosis, in the activation of proinflammatory transcription factor NFkB, along with its role in signalling pathways linking numerous biological events which include antiviral defence and cell growth, are clear indicators of the great potential that this protein may have in a variety of pathologies (Figure 7). The identification of PKR as a target of both conventional chemotherapeutic and novel drugs highlights the need to carry out translational studies with patients to validate its potential as a biomarker of important diseases like cancer and neurodegeneration. Moreover, the PKR deregulation showed in diseases like Alzheimer and inflammatory processes underlines the need to find applicable inhibitors of this kinase. Since PKR is expressed in almost all cells, its therapeutic potential could arise from the factors involved in its regulation. In fact, understanding the cellular factors and signals that regulate the role of PKR in the different diseases would be extremely valuable from a clinical point of view.

The present review has shown the importance to conduct continued basic molecular and preclinical studies. Further along the road, clinical studies could show how an application could be made to diagnostics and pharmacology. With personalised medicine looming in the future and the genetic and proteomic background of each patient determining specific approaches, PKR could play an extremely relevant clinical role.

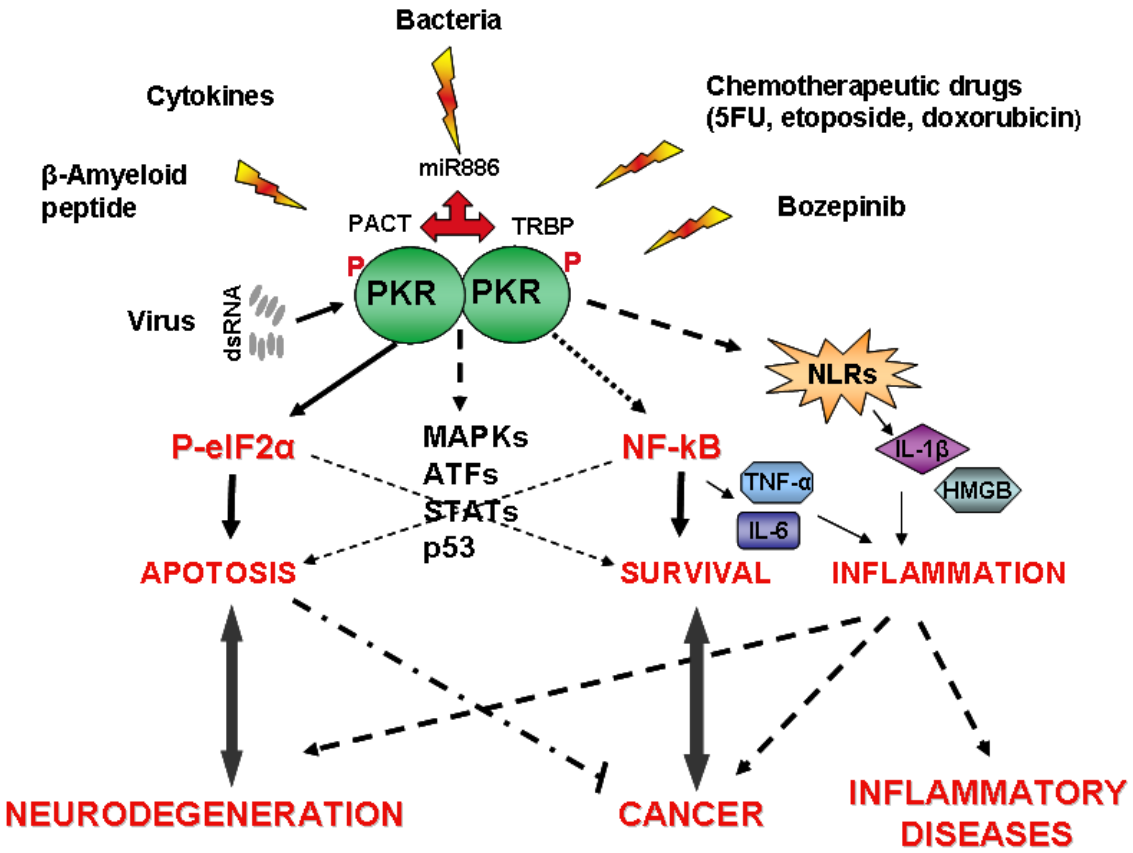


Figure 7. Representative diagram of PKR activation and modulation: pathological consequences of an unbalanced PKR activity. PKR is activated by a variety of cellular stresses including viral and bacterial infections, cytokines, and chemotherapeutic drugs. PKR activation involves its autophosphorylation, which triggers the phosphorylation of translation initiation factor eIF2α and the activation of the proinflammatory NF-kB transcription factor. Both effects contribute to induce apoptosis cell death or to induce cell proliferation depending on the stimulus and unknown factors. In addition, PKR is involved in inflammasome activation and cytokines liberation as IL-6, TNF α and HMGB among others. Moreover PKR is involved in various pathways that engage multiple genes, including MAPKs, ATFs, STATs, and p53, among others. Diverse PKR modulators have been identified, highlighting the PKR activator PACT, the inhibitor TRBP, and the microRNA mir886. An unbalanced effect induced by dysregulated PKR activity could contribute to neurodegeneration, cancer and inflammatory diseases.

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