Chapter

Phosphorylation of NF-κB in Cancer

Matthew Martin, Antja-Voy Hartley, Jiamin Jin, Mengyao Sun and Tao Lu

Abstract

The proinflammatory transcription factor nuclear factor-κB (NF-κB) has emerged as a central player in inflammatory responses and tumor development since its discovery three decades ago. In general, aberrant NF-κB activity plays a critical role in tumorigenesis and acquired resistance to chemotherapy. This aberrant NF-κB activity frequently involves several post-translational modifications of NF-κB, including phosphorylation. In this chapter, we will specifically cover the phosphorylation sites reported on the p65 subunit of NF-κB and their relationship to cancer. Importantly, phosphorylation is catalyzed by different kinases using adenosine triphosphate (ATP) as the phosphorus donor. These kinases are frequently hyperactive in cancers and thus may serve as potential therapeutic targets to treat different cancers.

Keywords: kinase, NF-κB, phosphorylation, post-translational modifications

1. Introduction

1.1 Basic nuclear factor-κB (NF-κB) family and signaling pathways

So what is NF-κB? In mammals, NF-κB is a collective term for a small family of dimeric transcription factors [comprising p65 (RelA) and RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2)]. All NF-κB proteins share a Rel homology domain (RHD), which is responsible for DNA binding and dimerization. Only p65, RelB, and c-Rel contain potent transactivation domains within sequences from C-terminal to the RHD. Therefore, p50 and p52 cannot act as transcriptional activators by themselves. Dimers of these two proteins have been reported to repress NF-κB-dependent transcription *in vivo*, most likely by competing with other transcriptionally active dimers. These proteins form homo- and heterodimers, and their activity is regulated by the canonical or alternative pathways described as following [1]. A simple diagram of canonical NF-κB signaling, which will be the focus of this chapter, is shown in **Figure 1**. The canonical pathway is activated by multiple stimuli, including proinflammatory cytokines (e.g., tumor necrosis factor, TNF; interleukin 1, IL-1), and the components of the bacterial wall (Lipopolysaccharide, LPS). Exterior signals lead to the phosphorylation and degradation of the inhibitory complex IκB, which is modulated by the IκB kinase (IKK), and its degradation allows for the release of the typical NF-κB

heterodimer, p65/p50, to translocate into the nucleus. NF-κB binds to its cognate DNA elements and can transcriptionally activate different target genes among which 200–500 genes have been implicated in cell survival/apoptosis, cell growth, immune response, and inflammation [2].

The alternative or noncanonical pathway is activated by the members of the TNF cytokine family, such as B-cell activating factor (BAFF), cluster of differentiation 40 ligand (CD40L), receptor activator of nuclear factor-κB ligand (RANKL), and lymphotoxin-β2 (LTβ2), and requires recruitment of the p52/RelB dimers to activate transcription. Firstly, activation of NIK (NF-κB-inducing kinase) leads to

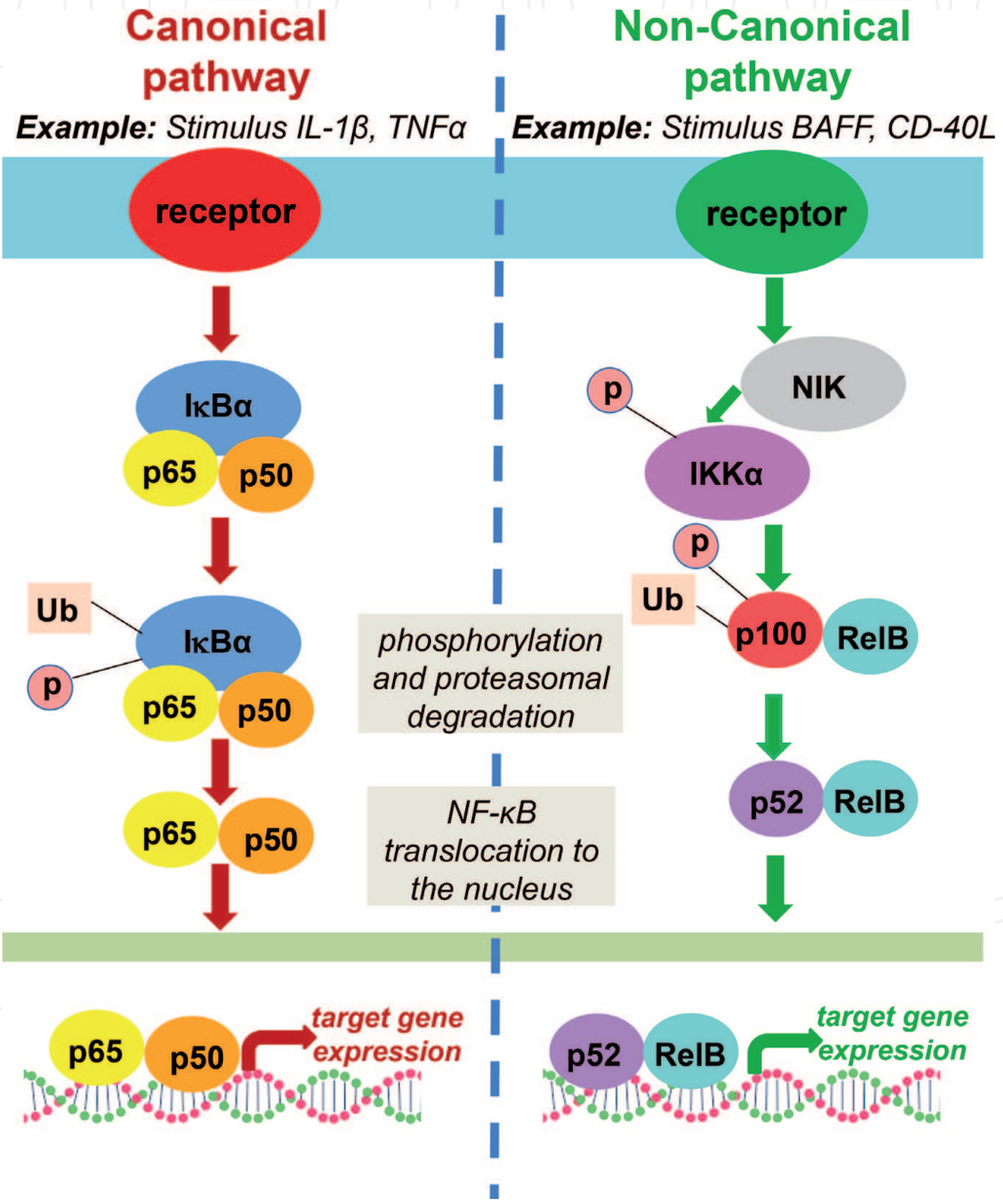

Figure 1.
Pathway of canonical and non-canonical NF-κB signaling. Under the canonical pathway of NF-κB signaling, activation of the NF-κB is initiated by a stimulus resulting in phosphorylation and subsequent proteasomal degradation of IκBα. This allows the release of the p65/p50 heterodimer into the nucleus, where they can bind to their cognate DNA elements and promote NF-κB target gene expression [1]. On the other hand, under noncanonical activation of NF-κB, NIK (NF-κB-inducing kinase) leads to activation of IKKα in this pathway. Subsequent phosphorylation of the NF-κB1 precursor molecule, p100, triggers partial proteolysis giving rise to p52, which preferentially dimerizes with RelB. This allows translocation of p52/RelB to the nucleus where they can bind to cognate DNA elements and promote gene transcription [1]. Figure adapted and simplified from Hoesel et al. [103].

activation of IKKα in this pathway. This event leads to the subsequent phosphorylation of the NF-κB1 precursor molecule, p100, and triggers partial proteolysis to give rise to p52, which preferentially dimerizes with RelB [1]. The p52/RelB heterodimer then translocates to the nucleus where they can bind to cognate DNA elements and promote gene transcription (**Figure 1**).

1.2 Important role of NF-κB in cancer

NF-κB was first discovered by Dr. Ranjan Sen in 1986 [3]. This family of transcription factors plays important roles in the regulation of apoptosis, proliferation, inflammation, and immune response in both normal and cancer cells. Generally, in normal cells, the central transcription factor NF-κB is transiently activated in response to certain stimuli. However, cancer cells usually exhibit sustained activation of NF-κB [4, 5] which significantly contributes to their survival. Moreover, NF-κB activity plays critical roles in many of the well-known "hallmarks" of cancer, via its regulation of target genes involved in tumor cell proliferation, suppression of apoptosis, activation of angiogenesis as well as induction of the epithelial-to-mesenchymal transition (EMT) phenotype, a critical step in metastasis [6]. Constitutively active NF-κB has been found in many types of cancer. For instance, in thyroid cancer, oncogenic proteins including "rearranged during transfection" (RET), "Rat Sarcoma" (RAS), and "v-Raf murine sarcoma viral oncogene homolog B" (BRAF) were shown to induce NF-κB activation, which in turn activated proliferative and antiapoptotic signaling pathways [7]. Moreover, in renal cell carcinoma (RCC), NF-κB is constitutively activated. The phosphorylated p65, a major subunit of NF-κB, exhibited a significant increase in the RCC samples compared with corresponding normal tissues [8]. Furthermore, Nogueira et al. showed that in glioblastoma (GBM), deletion of IκB showed a phenotype similar to that of epidermal growth factor receptor (EGFR) amplification in the pathogenesis of GBM. This was also correlated with low survival rates in affected patients [2].

Importantly, our laboratory also found that in colon cancer, NF-κB can be activated by the Y-box protein 1 (YBX1), a critical event correlated with increased colon cancer cell proliferation and anchorage-independent growth [9, 10]. Additionally, in pancreatic cancer, a mutant oncogenic KrasG12D (glycine to aspartic acid) mutation induced positive feedback loops of interleukin 1α (IL-1α) and p62 expression to sustain constitutive IKKβ (inhibitor of NF-κB kinase subunit β)/NF-κB activation [11]. In breast cancer, moderately elevated NF-κB led to chronic inflammatory conditions that result in some cells escaping immune surveillance [12]. Additionally, NF-κB activation was shown to upregulate the expression of cyclin D1, cyclin-dependent kinase 2 (CDK2), and c-Myc, which drives cell cycle progression and causes uncontrolled cell proliferation [13]. Moreover, in breast cancer, NF-κB has been shown to induce and stabilize the expression of EMT markers (Snail and twist-related protein1) [14], a pivotal process in tumor metastasis. In addition to the role of NF-κB in solid tumors as described thus far, Gasparini et al. has thoroughly reviewed the important tumorgenic role of NF-κB in hematological malignancies, including acute lymphocytic lymphoma (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chromic myeloid leukemia (CML), B lymphomas, diffuse large B-cell lymphomas (DLBCLs), Hodgkin's lymphoma, adult T-cell lymphomas (ATLL), anaplastic large-cell lymphomas (ALCL), and multiple myeloma, which will not be further discussed in this chapter [15]. Overall, these studies highlight a prominent role of dysregulated NF-κB in multiple aspects of cancer progression in both solid tumors and hematological malignancies.

2. Role of phosphorylation of NF-κB in cancer

2.1 Phosphorylation of the p65 subunit of NF-κB and the role of its kinases in cancer

Phosphorylation is a critical modification for NF-κB activation and plays an indispensable role in the regulation of its target genes. Moreover, as mentioned above, many of these target genes contribute to the hallmarks of cancer such as cellular proliferation, antiapoptosis as well as enhanced angiogenesis via vascular endothelial growth factor expression, among others [6]. Thus, understanding how phosphorylation of NF-κB contributes to these cancer phenotypes is a critical step in effectively limiting NF-κB activity [16]. Generally speaking, phosphorylation requires phosphorus which is supplied by the donor molecule adenosine tri-phosphate (ATP). Although several members of the NF-κB family of proteins are reported to be subjected to phosphorylation, p65 stands out as the most frequently modified subunit (**Table 1**). Furthermore, scientists have found that p65 can be phosphorylated by a variety of different kinases, some of which are themselves frequently overactive in cancer. For instance, p65 phosphorylation at serine 536 (S536) by IKKβ has been shown to be critical for TNFα-induced transformation of mouse epidermal cells [17]. Additional studies have also reported a role for p65 S536 phosphorylation in mediating expression of matrix metalloproteinase 1 (MMP-1) in lymphomas, wherein high MMP-1 expression correlated with lymphatic invasion and lymph node metastasis [18]. Another study with an immortalized prostate cell line, PNT1a, showed a role for phosphorylated S536 of p65 in cell motility and transformation [19].

As mentioned above, phosphorylation events on NF-κB are mediated by a variety of kinases. It is therefore unsurprising that the action of these kinases has been tightly regulated to maintain normal cellular function. However, deregulation

Known phosphorylation sites of p65	Cancers involved	Cell line discovered	References
S205	No cancers currently known	HEK 293 cells	[104]
T254	Breast cancer	BT20 and MCF-7 cells	[105]
S529	Breast cancer	HeLa cells	[33]
S536	Bone cancer	HeLa and BC-3 cells	[35]
S276	Head and neck cancers, breast cancer	HNSCC cells	[34]
S281	No cancers currently known	MEF cells	[106]
S311	No cancers currently known	HEK 293 and MEF cells	[107]
T435	No cancers currently known	SiHa cells	[108]
S468	No cancers currently known	HeLa cells	[109]
T505	No cancers currently known	NARF2 and Hs68 cells	[110]
S535	No cancers currently known	HeLa cells	[111]
S316	No cancers currently known	HEK 293 cells	[41]
S547	No cancers currently known	HEK 293 cells	[112]

Table 1.
List of known phosphorylation sites on the p65 subunit of NF-κB and their relationship to cancer.

of kinase activity can have detrimental downstream effects, which also involves the aberrant activation of NF-κB and its target genes to promote a cancer phenotype. Several kinases have been shown to have critical roles in the regulation of the p65 subunit of NF-κB. For example, glycogen synthase kinase 3 beta (GSK3β) and TRAF-associated NF-κB activator TBK1 ((TANK)-binding kinase 1) have been shown to be critical activators of NF-κB signaling [20, 21] by targeting p65 for phosphorylation on S536. This phosphorylation leads to enhanced NF-κB transactivation both *in vitro* and *in vivo* [22–24]. Another well-known kinase involved in modifying p65 is protein kinase A (PKAc). PKAc is typically activated following IκBα-degradation, leading to PKAc-mediated phosphorylation of p65 on S276 [25]. This phosphorylation event causes recruitment of histone acetyltransferases including cAMP response element-binding (CREB)-binding protein (CBP) and p300. The net effect is displacement of p50-histone deacetylase (HDAC)-1 complex from DNA, which increases p65 transactivation ability [26, 27]. Other kinases can also phosphorylate p65 at S276. These include mitogen- and stress-activated protein kinase-1 and 2 (MSK1, 2), proto-oncogene serine/threonine-protein kinase PIM-1 (PIM-1), ribosomal s6 kinase (RSK) p90, and protein kinase C α (PKCα) [28–31]. Moreover, casein kinase II (CK2), which phosphorylates p65 on S529, has been implicated in breast cancer [32, 33]. Another study demonstrated a role for p65 S276 phosphorylation by protein kinase A (PKA) in promoting a malignant phenotype in head and neck squamous cell carcinoma (HNSCC). Here, the authors found that S276 phosphorylation was prevalent in the nucleus of HNSCC samples but cytoplasmic in normal mucosa. Furthermore, this TNF-α-induced nuclear p65-S276 phosphorylation was significantly inhibited by the PKA inhibitor H-89, which in turn suppressed NF-κB activity, target gene expression, cell proliferation, and induced cell death via G1/S phase arrest [34]. Other p65-targeting kinases implicated in cancer include cell division protein kinase 6 (CDK6) and PIM-1, which phosphorylate p65 at S536 and S276, respectively [29, 35]. Both PIM1 and CDK6 have been shown to be overexpressed in a variety of cancers including hematological cancers, prostate cancer, pancreatic cancer, gastric cancer, head and neck cancer, liver cancer, glioblastomas, medulloblastomas, colon cancer, and lung cancers [36–39]. However, their exact roles in regulating p65 phosphorylation in these cancers are yet to be understood. In gastric cancer cells, Aurora Kinase A (AURKA) was also shown to phosphorylate S536 in *in vivo* and *in vitro* models [40] whereby overexpression of AURKA induced a significant increase in NF-κB p65 and phospho-p65 (S536) protein levels. Interestingly, protein kinase C δ (PKCδ), a member of novel PKC isoforms, has also been implicated in a number of cancers including breast, pancreatic, prostate, and melanoma tumor cells but has been shown to regulate p65 transactivation in a phosphorylation-independent manner [41]. Our laboratory has also recently reported a novel phosphorylation site on S316 of p65, a modification mediated by the kinase CKII [41]. We showed that S316 phosphorylation was necessary for NF-κB activation and target gene expression. Collectively, these examples indicate the importance and sophistication of p65 phosphorylation and their corresponding kinases in regulating NF-κB signaling in the context of cancer.

2.2 Importance and effects of phosphorylation of p65 in modulating chemoresistance

Several studies have indicated a role for NF-κB hyperactivity in the development of resistance to chemotherapeutics via downregulation of antisurvival and upregulation of prosurvival target genes and pathways [42–44]. In one study for example, gemcitabine-resistant pancreatic cancer cells were rendered sensitive to gemcitabine upon knockdown of p65 [42]. These and other accounts of NF-κB-mediated

chemoresistance have been extensively reviewed by others such as Li, Sethi, and Godwin et al., which will not be further discussed in this chapter [45, 46]. However, the specific contribution of dysregulated p65 phosphorylation to chemoresistance is less well understood and requires further exploration. Nonetheless, a few reports suggest that upstream kinases involved in chemoresistance can modulate p65 phosphorylation levels in this context. For instance, siRNA-mediated depletion of IKKα in HT1080 human fibrosarcoma cells was shown to decrease phosphorylation of p65 in response to doxorubicin, thus severely impairing the ability of doxorubicin to initiate NF-κB DNA-binding activity. These findings suggest that IKKα plays a critical role in NF-κB-mediated chemoresistance in response to doxorubicin and potentially serves as a therapeutic target for improving chemotherapeutic response [47]. Other studies have shown that p65, in a hyperphosphorylated state, can be correlated with resistance to thymidylate synthases and irinotecan in stomach and colon cancers, respectively [44, 48, 49]. Doxorubicin resistance in lung cancer has also been correlated with p65 S536 phosphorylation states [47]. Additionally, multiple myelomas have exhibited increased p65 S536 phosphorylation within melphalan- or doxorubicin-resistant cells [50].

3. p65 modifying kinases as potential therapeutic targets

3.1 Current therapeutics used to treat cancers with constitutive NF-κB activity

The NF-κB pathway is widely considered an attractive therapeutic target in a broad range of cancers. Yet, despite the efforts to develop NF-κB inhibitors, none has been clinically approved. This is largely due to immune-related toxicities associated with global NF-κB suppression [51]. Furthermore, the high complexity of the NF-κB signaling network presents another unique challenge for developing specific NF-κB inhibitors. To further complicate matters, some standard anticancer agents can inadvertently activate the NF-κB pathway via induction of proinflammatory cytokines such as IL-1β and TNF-α and cellular stressors such as reactive oxygen species (ROS), or by activating DNA-repair mechanisms [52]. Finally, constitutive NF-κB activity can also be achieved via secreted cytokines and chemokines from inflammatory cells within the tumor microenvironment [52]. Taken together, consideration of all these factors is imperative when strategizing the development of the most effective and least toxic anticancer agents.

Currently, the use of NF-κB inhibitors has mainly been combined with other agents [47, 50]. Some of these combinatorial therapies have shown promising effectiveness and have been made it as far as the clinical trial phase. For example, combination of irinotecan with the proteasome and NF-κB inhibitor bortezomib was shown to increase sensitivity of colon cancer cells to irinotecan [53]. A separate study showed that bortezomib could sensitize non-small cell lung cancer (NSCLC) cells to sodium butyrate, which acts to inhibit histone deacetylases [54]. Moreover, several clinical trials testing the efficacy of inhibitors against IKK to target solid tumors have been undertaken. For example, perturbation of IKKβ with the inhibitory ML120B led to synergistic enhancement of vincristine cytotoxicity in lymphoma. These results implicate IKK disruption using inhibitors as a useful adjunct therapy with standard chemotherapeutics. Other attempted trials using IKK inhibitors, such as CHS-828, EB-1627, and IMD-1041, as single or combinatorial agents unfortunately produced toxicity concerns for patients [55–57].

Other examples of combinatorial therapies include the use of NF-κB inhibitors Bay11-7082 and sulfasalazine in combination with more commonly used

chemotherapeutics such as 5-fluorouracil and cisplatin to synergistically reduce colon cancer cell growth [58]. Other indirect means of targeting NF-κB such as inhibition of upstream kinases have also shown promise. For instance, one study using pancreatic cancer cells showed that inhibition of GSK3β by a small molecule inhibitor reduced phosphorylation of p65 at S536 resulting in decreased NF-κB activity and cell growth [59]. Another study with the chemical compound ursolic acid showed reduced p65 phosphorylation via inhibition of IKKβ, which impaired overall cell growth in leukemia cell lines [60]. Other studies with proteasome inhibitors, including Tosyl phenylalanyl chloromethyl ketone (TPCK) and Tosyl-L-lysyl-chloromethane hydrochloride (TLCK), demonstrated that not only do these inhibitors target IKKβ, but they were also able to reduce overall phosphorylation levels of NF-κB [61, 62]. In summary, these studies suggest there may be many benefits to targeting hyperactive NF-κB signaling and, in particular, the kinases that regulate NF-κB in various cancers.

3.2 Benefits and pitfalls for targeting kinases in cancers

The development of small-molecule kinase inhibitors for the treatment of cancer has continued to be of intense interest. Notably, many inhibitors have received FDA approval with approximately another 150 are in preclinical and clinical phase trials. Despite these important advances, many factors have confounded the clinical efficacy of these kinase-targeted drugs including the challenges of tumor heterogeneity and microenvironment as well as the emergence of mutations that confer drug resistance. Another major challenge of kinase inhibition is that of the development of adverse side effects. Some classic examples of this include dermatologic complications and cardiotoxicity associated with inhibition of EGFR and vascular endothelial growth factor receptor (VEGFR), respectively. Furthermore, there is an urgent need to develop relevant models of resistance in response to kinase inhibitors in efforts to overcome this resistance via potential synergistic combinatorial therapies.

Another critical issue facing clinical trial design with kinase-targeted agents is that of determining the types of tumors that are most likely to respond to specific kinase inhibitors and thus identify the subsets of patients who will likely benefit from these treatments. To combat this issue, many studies have been dedicated to identifying certain "kinase dependencies" in cancer cells that would make them more susceptible to inhibition. These so-called dependencies are primarily based on the existence of constitutively activate kinases achieved by gene mutation, amplification, or fusion. Among the potential approaches to identifying signatures of kinase dependency are proteomic profiling, next-generation sequencing and various applications utilizing phospho-specific antibodies against numerous specific kinase substrates. Additional mechanisms of kinase dependency include impairment of the function of phosphatases, the negative regulators of phosphorylation as is the case with mutations in the phosphatase and tensin homolog (PTEN) tumor suppressor gene. The consequence of *PTEN* loss is signal propagation through downstream kinases such as Akt. Moreover, growing evidence from isogenic human and mouse models also suggests that this type of indirect avenue of kinase dependency may be analogous to direct, activating mutations in the kinases themselves.

Finally, there are also cases in which the beneficial effect of a kinase inhibitor is counteracted by an additional genetic lesion in a compensatory signaling pathway. Therefore, studies to identify such secondary events are urgently needed. Taken together, these evidences underscore the critical need to optimize the use of kinase inhibitors against cancers by continued detailed molecular characterization

of tumor tissues. It will be critical to develop new compounds that circumvent acquired resistance to the first-generation kinase inhibitors for patients with refractory disease.

3.3 Cutting edge therapeutics for treating cancers with constitutively active kinases

Since the mid-1970s, numerous studies have highlighted a crucial role for kinases in promoting tumorigenesis and metastasis [63–67]. This is of no surprise, since a clear majority of protein kinases promote critical cellular functions pertinent to cancer progression, including cell proliferation, survival, and migration [68–72]. Hence, targeting mutated, overexpressed, or hyperactive kinases represents an important and promising clinical niche for developing drugs for cancer therapy [67, 73, 74]. Interestingly, inhibitors against kinases account for approximately 25% of all current drug discovery efforts. So far, ~37 inhibitors with activity targeted to one or multiple kinases have been approved for clinical use [67, 75]. These range from highly selective monoclonal antibodies to more broad-spectrum synthetic or natural small molecules that have achieved a significant increase in patient survival rate in cancers [67]. For example, treatment with imatinib and dasatinib, which are highly potent and selective tyrosine kinase inhibitors against BCR-ABL, produces more favorable outcomes compared to conventional cytotoxic therapy for patients with chronic myeloid leukemia (CML) [76–78]. Similarly, broad-spectrum inhibitors have also been used with great success. For example, CHIR-258, which targets multiple kinases, inhibits VEGFR, platelet-derived growth factor receptor (PDGFR), FMS-like tyrosine kinase-3 (FLT-3), mast/stem cell growth factor receptor (c-Kit), and fibroblast growth factor receptor (FGFR) in multiple myeloma patients and is most effective for killing tumors harboring the translocation t [4, 14] (p16.3;q32.3) with increased expression and activating mutations of FGFR3 [79–81].

Some other major inhibitors to enter the market include those targeting key oncogenic kinase drug targets such as ERBB2 (e.g., afatinib), HER2 (e.g., trastuzumab), and VEGFRs (e.g., sorafenib, a small molecule inhibitor used for the treatment of renal cell, liver. and thyroid cancers) [82–87]. Another successful targeting strategy has been the use of a monoclonal antibody against VEGFR (bevacizumab). Bevacizumab is used in combination with chemotherapy to treat patients with metastatic colon cancer, and its widespread use has resulted in significant improvement in survival outcomes [88]. Other examples include inhibitors against Kit, PDGFRs, proto-oncogene tyrosine-protein kinase Src (SRC), mechanistic target of rapamycin and FK506-binding protein 12-rapamycin-associated protein 1 (mTOR), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), serine/threonine-protein kinase B (PKB or Akt)—Raf (BRAF), and epidermal growth factor receptor (EGFR) (e.g., cetuximab, panitumumab), all of which act to activate significant tumor cell signaling pathways such as NF-κB [67, 89]. For instance, several FDA-approved kinase inhibitors, although not perceived as direct NF-κB inhibitors, have been shown to suppress NF-κB signaling [89]. These include inhibitors targeting EGFR in breast and lung cancer, Akt in breast cancer, GSK-3β in pancreatic cancer, NIK in melanoma, BRAF in multiple myeloma, and IKK in brain and liver cancer [89–91]. Specifically, GSK2118436, PLX4720, sorafenib, and PLX4032 are all drugs which are currently being used to target $B\text{-}Raf^{V600E}$ in advanced cancers with elevated NF-κB activity [73, 92–94]. Finally, small molecule inhibitors targeting Akt, which include perifosine, GSK690693, VQD002, and MK2206, are also being tested clinically [95, 96].

In summary, these studies highlight the importance of inhibition of distinct kinase signaling pathways as a means of minimizing cytotoxic effects on non-cancerous cells, thus bestowing selective killing of tumor cells and improving patient clinical outcomes.

4. Conclusion and future directions

In summary, this chapter highlights a significant role of NF-κB phosphorylation in driving the initiation and progression of several cancers as well as chemoresistance to first-line therapies. Furthermore, we emphasized the relationship between p65 phosphorylation and the role that constitutively active kinases play in promoting the cancer phenotype, via utilization of ATP as the phosphate donor. Finally, we underlined the well-established therapeutic potential of targeting these kinases in the treatment of various cancers. Despite these encouraging data, we acknowledge that the difficulties with drug resistance and toxicity continue to present critical challenges for the use of kinase inhibitors in both clinical and experimental oncology. Furthermore, issues related to the inadequate understanding of the selectivity of the kinase inhibitors have also plagued the successful clinical utility of these inhibitors. Nevertheless, a key challenge for overcoming this enigma is to identify the most efficacious, complementary, and least toxic combinations of kinase inhibitors for targeted cancer treatment [97, 98]. This will likely lead to the development of multimodal treatment initiatives that evade the treatment-related drug resistance. Finally, it is well known that cancers have heterogeneous populations of cells, and these may differentially contribute to chemoresistance [99, 100]. To address this, many efforts are underway to eliminate cancer stem cells as main culprits of this intrinsic heterogeneity, which will undoubtedly improve our understanding of better drug design and efficiency [101, 102].

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