

Exposition of Protein Kinase Targeted Nanoplatfoms: An Extensive Review

Running Title: A Bird's-Eye View of Protein Kinase Targeted Nanoplatfoms

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Abstract Protein kinases play a prominent role in transferring a phosphate group from ATP (adenosine triphosphate) to serine, tyrosine, or threonine residues. Mutation and dysregulation in their activity lead to an imbalance in the health causing diseases like cancer. Protein kinase inhibitors have proved to be more effective in addressing such impairment caused by the dysregulation of protein kinases. Several protein kinase inhibitors like antibodies and small molecules have been approved by the regulatory bodies like US FDA, and many of them are being developed and expected to enter the clinical trials and subsequently to the market in the near future. However, targeting such kinase inhibitors to the desired site of action remains a challenging process. The current review focuses on the applicability of Nano platfoms for targeting protein kinase inhibitors to the desired site of action and thus enhancing the therapeutic efficacy of the drug molecules against cancer. Besides, it also gives a brief idea to the

readers about the imaging nanoplatfoms investigated for the detection of protein kinase inhibitor activity along with the list of ongoing clinical trials pertaining to protein kinase inhibitors.

Keywords Protein Kinase Inhibitors, Nanoplatfoms, Cancer, Potential Target

1. Introduction

The term 'kinases' has been an interesting topic for the scientists, since the identification of phosphorylation reaction in the glycogen metabolism. Protein kinases are a group of enzymes that modify proteins by means of phosphorylation (addition of terminal phosphate moiety of adenosine triphosphate (ATP) to threonine, tyrosine and

serine residues) [1]. This phosphorylation induces a change in the target protein by monitoring the signaling pathways and thus alters the functionality of target proteins [2]. Kinases are usually involved in several cellular functions like metabolism, differentiation, survival and cell cycle regulation. More importantly, they also regulate the phosphorylation of target proteins. Any alteration in this regulation leads to diseased condition.

It was reported that amongst the available 518 protein kinase genes, 244 genes map to the cancer amplicons [1]. For instance, *c-Src* (non-receptor tyrosine kinase) was the first proto-oncogene identified in 1976 [3]. The kinases can be classified into two broad categories based on the amino acid sequences of the domains and substrate specificity. The earlier class depends on the sequences of the catalytic domains [1]. While the later involves the kinases classified based in the amino acids (mainly serine-threonine and tyrosine) they phosphorylate [4]. The kinases are further divided as receptor kinases and non-receptor kinases. Receptor's kinases are transmembrane proteins having a catalytic intracellular domain and ligand-binding extracellular domain. While, the non-receptor kinases are found in cytosol, inner surface of plasma membrane and nucleus but, lack the transmembrane domains [5].

The dysregulation and mutations of protein kinases involve multiple mechanism like relocation, over-expression, point mutations, fusions and dysregulation of the upstream signaling [6,7]. From this we can comprehend that, protein kinases play a prominent role in human diseases and have turned out to be one of the prominent drug targets in the recent past [8]. Understanding the kinases also helps in the development of targeted and personalized therapies for diseases like cancer. Cancer describes any illness characterized by the uncontrolled proliferation of abnormal cells. Due to the complications associated with cancer, it is well-known that cancer is not just one illness but instead a conglomerate of many ailments [9].

BRAF, EGFR, and PIK3CA are the key drug targets for oncogenic kinases [10,11]. Furthermore, the kinase inhibitors like dasatinib and imatinib have produced more favorable results when compared with the conventional cytotoxic therapy [12,13]. Due to the improved efficacy of these inhibitors in the clinical environment, several small molecule kinase inhibitors targeting the kinome members like epidermal growth factor receptor (EGF), vascular EGF (VEGFRs), *c-ABL*, mTOR, PDGFRs, ERBB2 and SRC are approved by the U.S. FDA. Most of them target the ATP-binding site [14] and very few target the allosteric sites [15]. Consequently, inhibiting the kinase activity, induces several anti-proliferative mechanisms in the patients and thus, helps in the clinical remission of cancer. Currently, the FDA has approved 52 small molecules for protein kinase inhibition and most of them are orally effective [16]. However, the problems such as compromised efficacy, toxicity, and drug resistance,

present critical hurdles in both experimental and clinical oncology [17,18].

Nanotechnology serves the need of the hour in addressing such problems faced in achieving desired efficacy and in overcoming the challenges of drug resistance. In the recent past, the nanotechnology-based therapeutic platforms have exhibited a significant role in delivering the therapeutic moieties to the desired site of action. Owing to their small size (50-200 nm), the nanoparticles (NPs) display specific optical, catalytic and electronic properties, which can be effectively employed for the drug delivery [19]. Various diagnostic and therapeutic agents have been encapsulated as a cargo in the NPs and administered them via different routes to treat cancer. NPs can carry multiple payloads for immune evasion, targeted transport and enhance the favorable release kinetics of the drugs at the target site [20]. In the present review, we have focused on the role of nanoparticles in delivering the therapeutic moieties and their role in functioning as an imaging platform by carrying suitable contrast agents is being highlighted.

2. Types of Protein Kinase Inhibitors

Protein Kinase Inhibitors (PKIs) stand efficacious in treating the cancer specially targeting the specific mutations which drive the tumorigenesis. Based on the ability to catalyze the process of transferring the ATP's terminal phosphate moiety to substrates containing serine, tyrosine or threonine residue. Several types of classification have been described earlier by reviewers. Herein, we have described the types of kinase inhibitors (Table 1) [18].

Table 1. Different types of Protein kinase inhibitors and their mechanisms [18]

Type of Kinase Inhibitor	Mechanism	Example
Type I	Binds to the active conformation of ATP-binding pocket by competing for the substrate	Gefitinib, Ruxolitinib
Type II	Binds to the inactive enzyme form of DFG-Aspartate out conformation of protein kinase	Axitinib, Imatinib
Allosteric Inhibitor	Binds to the site available next to the ATP-binding pocket to allow both allosteric inhibitor and ATP to bind with the protein simultaneously	GnF2, Trametinib
Substrate Directed Inhibitor	It offers selectivity against the targeted kinases by undergoing reversible interactions outside the ATP pocket	ONO12380
Covalent Inhibitor	Binds to the protein kinase target covalently	Ibrutinib, Afatinib

3. Therapeutic Nano Platforms Targeting Protein Kinase Inhibitor

3.1. Antibodies and Small Molecule Inhibitors

Several approaches have been proposed and developed for targeting the protein kinases. The most popular ones use the antibodies or small molecules to block the interaction between kinases and the substrates or to hinder enzymes catalytic activity by binding with the enzyme's ATP binding site. Few examples include HER2 inhibitor like trastuzumab used to treat breast cancer [21], and EGFR inhibitor like cetuximab and gefitinib used to treat colorectal and lung cancer [22]. However, the major challenge is delivering these bio-actives to a specific target without causing toxicity issues. Because of this, though many protein kinase inhibitors (PKI) are being successfully developed, only 5% of them entering the clinical trials are getting market approval. This hurdle can be effectively addressed by the nanotechnology [23]. For instance, liposomes were used in a study to deliver cabozantinib (tyrosine kinase inhibitor) [24]. The liposomes enabled the sustained release of anticancer agent and enhanced the targeting of tumour region. The cytotoxicity studies showed that the small molecule loaded liposomes exhibited higher cytotoxicity on renal cancer cells when compared to the plain drug. *In vivo* studies on renal cell carcinoma induced nude mice proved that the liposomes were more accumulated in the tumour, resulting in lowered toxicity and thereby enhanced the tumour inhibition and reduced the dose related adverse effects. Similarly, lapatinib a dual inhibitor of EGFR and HER2 was encapsulated in lipoprotein NPs and targeted against breast cancer [25]. The *in vivo* studies showed that the lapatinib loaded NPs accumulated in tumour region via prolonged circulation and EPR (enhanced permeation and retention) effect. The drug loaded NPs arrested the cancer cells growth in G0/G1 phase, suggesting the potential of the nanoplatfom against breast cancer.

3.2. Protein Kinase Pathways as a Potential Target

There are several studies which have employed the nanoparticles to deliver the PKIs to target specific kinase pathways in the tumour cells [26]. The MAPK (microtubule associated protein kinase) signaling pathway which is dysregulated in many of the cancers was targeted in a study [27], via nanoparticle-mediated targeting. The hexadentate PLGA polymeric NPs were chemically

conjugated with PD98059 (MAPK inhibitor). The cellular uptake studies showed that the NPs were taken up by the tumour cells via endocytosis and *in vitro* showed that the NPs released the PD98059 in a sustained manner. This inhibited the phosphorylation of downstream signal (extracellular) regulated kinase. The *in vitro* cytotoxicity studies showed that the MAPK inhibitor loaded PLGA NPs not only inhibited the proliferation of lung and melanoma cancers, but also induced apoptosis. Furthermore, the *in vivo* studies performed on the melanoma-bearing mice demonstrated the antitumour efficacy of the NPs [27].

In another study, PI3K (phosphatidylinositol-3-kinase) signalling pathway was targeted using PLGA NPs [28]. The LY294002 (PI3K inhibitor) loaded PLGA-NPs were formulated using emulsion-solvent evaporation method. The drug release studies showed the sustained release of the PI3K inhibitor loaded NPs and the release kinetics was dependent on the type of cell. The cytotoxicity studies performed on three cell lines (melanoma, lung and breast carcinoma) proved that the NPs evidently inhibited the viability of lung and melanoma cancer cells (cell viability being 25% and 27% respectively after 48 h of incubation). The PI3K pathway inhibition leads to the inhibition of Akt phosphorylation which in turn results in induction of apoptosis and inhibition of the tumour proliferation as shown in Figure 1. The breast cancer cells exhibited more tolerance to the NPs with a viability of 122%. Further, the authors tested the ability of NPs to inhibit angiogenesis by using zebrafish tumour xenograft model. The results demonstrated that the loaded NPs could inhibit the angiogenesis completely from subintestinal vessel. Similarly, another study explained the role of silver and gold NPs coated with antibodies in regulating the membrane receptor internalisation to target ErbB2 receptor (tyrosine kinase receptor) [29]. The gold and silver NPs of different size were engineered with Herceptin (Her) to enable ErbB2 receptor targeting and studied the role of NPs size in activation and binding of the membrane receptors. The results showed that the particles of size 50 nm were optimal for the uptake by cells possibly due to the balance between receptor mediated endocytosis and multivalent crosslinking of the membrane receptors. Further, there was a significant reduction in the MAPK and Akt activation. However, it was also observed that the Her-NPs increased caspase-3 and 9 cleavage, resulting in twofold enhancement in the cell death in comparison with Her alone.

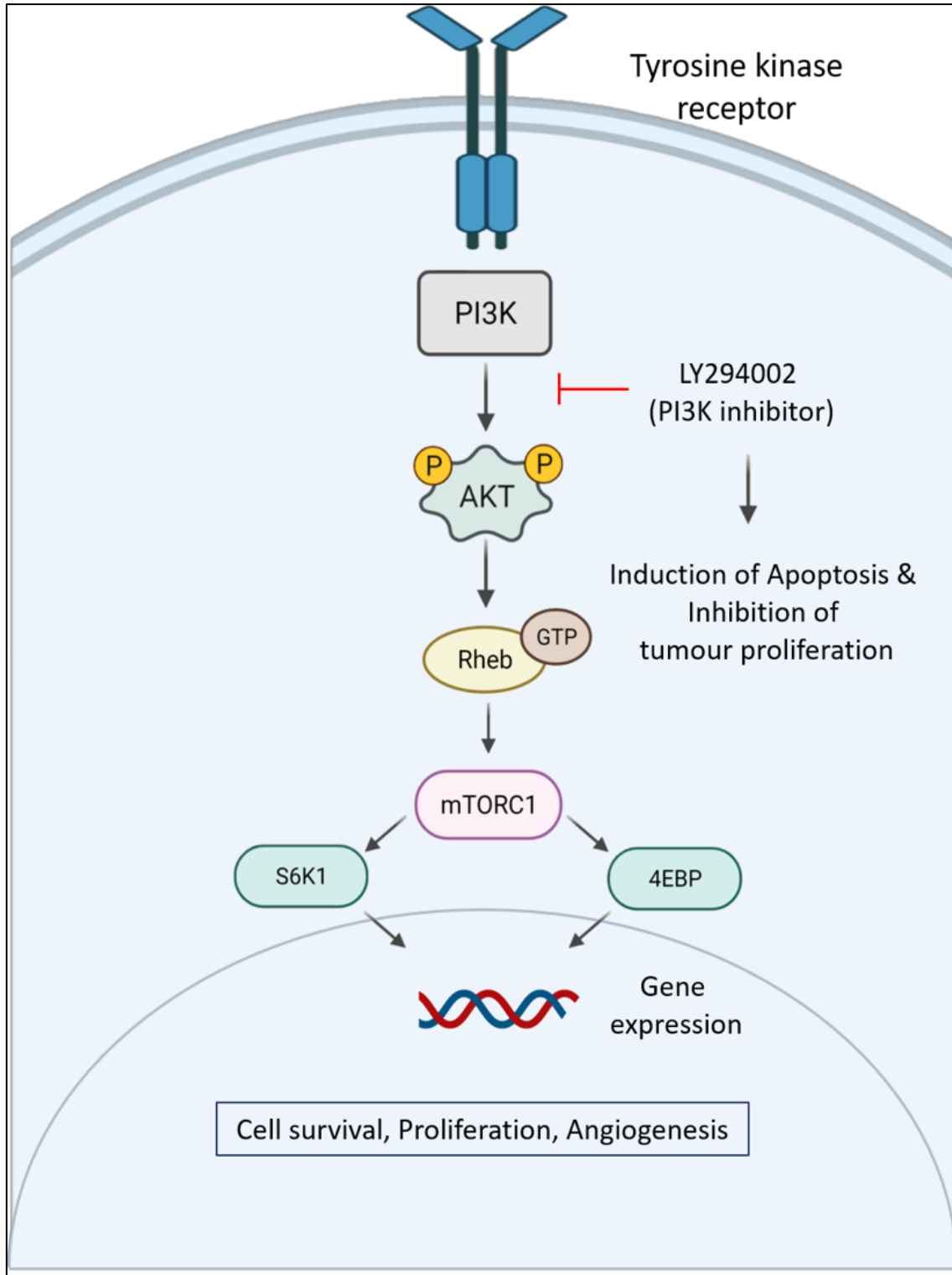


Figure 1. PI3K pathway inhibition

3.3. Protein Kinases-Based Combination Therapy

The approach of combination therapy usually involves the use of two or more therapeutic moieties so as to enhance the efficacy of drug or to overcome the drug resistance. This approach provides a scope to lower the dose of individual drugs and thus minimises the possible side effects [30]. In this section we have discussed the various possibilities of nano-based combination therapies involving the protein kinase inhibitors.

3.3.1. Protein kinases with immunotherapy

In several cases, antibodies are employed along with the PKIs loaded inside the nanoplatfoms to target the kinases for anticancer activity [31,32]. While in some cases, this combination is used to modulate the tumour microenvironment [33]. For instance, in one of the studies, sunitinib was encapsulated in PLGA nanovehicles to synergise the therapeutic efficacy of tyrosine related protein-2 nanovaccine (Trp2) in treating melanoma. The results demonstrated that the nanosystem not only enhanced the cytotoxic T cell infiltration but also, induced a shift in the expression of cytokine from Th2 to Th1. In addition, the inhibition of pathways like AKT and Stat3, may decrease the tumour evasion and induce apoptosis in tumour cells [34]. In another study, sunitinib was combined with TLR agonist (Toll-like receptor) nanovaccine along with PD-L1 (programmed death-ligand 1) antibody based on the hypothesis that the modulation of tumour microenvironment will augment activation of CD8 T cell and eventually block their inhibitory signalling. Therefore, the combination of sunitinib, PD-L1 and nanovaccine could reduce MDSCs and M2 macrophages along with the upregulation in the activation of CD8 T cells inside the tumour. The therapeutic efficacy of the developed nanosystem was evaluated *in vivo* using B16F10 (melanoma) and MB49 (bladder carcinoma) murine models. The results showed a decrease in the tumour volume and also abrogated the tumour-associated immune suppression [35]. Similarly, in another study, EGFR associated drug resistance in NSCLC (non-small cell lung cancer) was overcome by developing simvastatin and gefitinib loaded liposome using PD-L1 as a targeting ligand. The novel nanobody remodelled the tumour microenvironment leading to the suppression of TGF- β and consequently induced an antiangiogenic effect along with the upregulation in reactive oxygen species (ROS) [36].

3.3.2. Protein kinases with other biological actives

A lot of studies have been performed combining the PKI with natural substances having effects on the human metabolism. For instance, the combination of sorafenib and ursolic acid was delivered via mesoporous silica particles (MSNPs) coated with asialoglycoprotein receptor targeting agent like lactobionic acid and pH sensitive chitosan against hepatocellular carcinoma [37].

Ursolic acid helped in down regulating the EGFR (cell proliferation) and VEGFR (tumour angiogenesis) along with anticancer activity [38]. The nanocomplex exhibited pH-responsive sustained release profile and displayed synergistic cytotoxicity against the liver cancer at non-toxic concentrations. Furthermore, the nanocomplex down-regulated the overexpression of VEGFR2 and EGFR proteins and enhanced the cellular apoptosis. Similarly, a combination of sunitinib and curcumin was loaded iron oxide NPs and coated with bovine serum albumin (BSA) [39]. Curcumin had an anti-metastatic and anti-invasion effect [40,41] and being widely used in the nanomedicines recently. The BSA coating was given to prolong the circulation time, lower the immunogenicity and to achieve binding of the nanoformulation with specific binding domains. The *in vivo* pharmacokinetic studies performed demonstrated that the dual drug loaded nanoformulation efficiently delivered anticancer agents to tumour site and maintained the ratios of both the drugs concentration to yield the optimal synergistic effect.

4. Imaging Nano Platforms for Protein Kinase Inhibitor

Protein kinases control a number of cellular processes in a highly regulated sequence of phosphorylation reactions [42]. To develop the effective therapeutic platforms, it becomes important to understand the molecular mechanisms. Noninvasive detection of such changes helps in monitoring the progress of treatment strategies and also enables to improve the approach. The focus of kinase inhibitors is to target the over expressed growth factors and suppress them [43]. Usually, such an inhibition either involves the blockage of ligand binding domain using antibodies/peptides or by inhibiting the tyrosine kinases at the ATP binding site using small molecule inhibitors [44]. Developing an imaging platform to assess the pharmacological density of such clinical agents aids in enhancing the treatment strategies.

4.1. Near-Infrared (NIR) Imaging

Molecules absorbing in the NIR (700-1000 nm) region, can be used effectively to visualize the *in vivo* molecular targets and investigate them as most of cells generate NIR fluorescence to a little extent. Thus, NIR fluorescence enables quantitative and extremely sensitive analysis of the enzyme activity in the cultured cells [45]. In a study, protein-phosphorylation-responsive, biocompatible and cell-permeable polymeric NPs were developed for visualizing the protein kinase-A activity in the cells using Cy5.5 an NIR fluorochrome [46]. The polymeric NPs have a protein kinase-A specific peptide motif and are easily fabricated by self-assembly of the PIC (polyion-induced complex). The CY5.5 fluorochrome was chemically coupled with the PIC NPs and displayed

minimum intensity of fluorescence in quenched state, due to the short distance amongst each fluorochrome [47,48]. But in the phosphorylated state the PIC NPs dissolve and result in the dequenching of the NIR fluorescence. This activity of protein kinase-A was assessed in the CHO-K1 cells which overexpress the enzyme and the results confirmed that the developed nanoplatform clearly demonstrated the protein kinase activity.

4.2. Magnetic Resonance Imaging (MRI)

MRI is a specific tool used for analyzing the biological specimens non-invasively at high temporal and spatial resolution [49]. Most of the MRI sensors incorporate synthetic contrast agents in supramolecular assemblies or complex structures [50], which pose several limitations in designing, producing and delivery such structures *in vivo*. In a study, protein-based NP MRI sensors were developed to overcome such limitations of complex structures [51]. The new sensors developed were derived from the ferritin (Ft) protein and were self-assembled in the cells homing DNA sequences in specific combinations to detect the PKA activity, the study provided a proof of concept for the *in vitro* applicability of these protein-based MRI sensors and could serve as a potential imaging platform for the *in vivo* applicability in the near future.

4.3. Dark-Field Imaging

The detection of single particle (SP) based on the signals emitted by isolated individual particles has proved to be an effective approach in enhancing the sensitivity of imaging. Among the several techniques of SP analysis like fluorescent, optical and electrochemical based strategies [52], SP enumeration (SPE) via dark-field imaging using noble metal nanoparticles like gold NPs, is more explored these days for bio detection, due to the enhanced signal resolution. One of the studies utilized this approach of SPE to detect kinase activity via PK-mediated phosphorylation reaction [53]. This phosphorylation leads to transfer of the biotin moiety to immobilized substrate peptide from γ -biotin-ATP [54]. Biotinylated peptides help in the adsorption of gold NPs through specific interaction between biotin and streptavidin. With the change in gold NP count in dark field microscope, protein kinase activity (PKA) can be analyzed quantitatively along with high sensitivity [55]. The schematic illustration of the PKA is represented in Figure 2. Therefore, SPE strategy displays an excellent ability to quantitatively screen the kinase inhibitor and also to probe the fluctuation of chemical-induced protein kinase-A activity in the cell lysates.

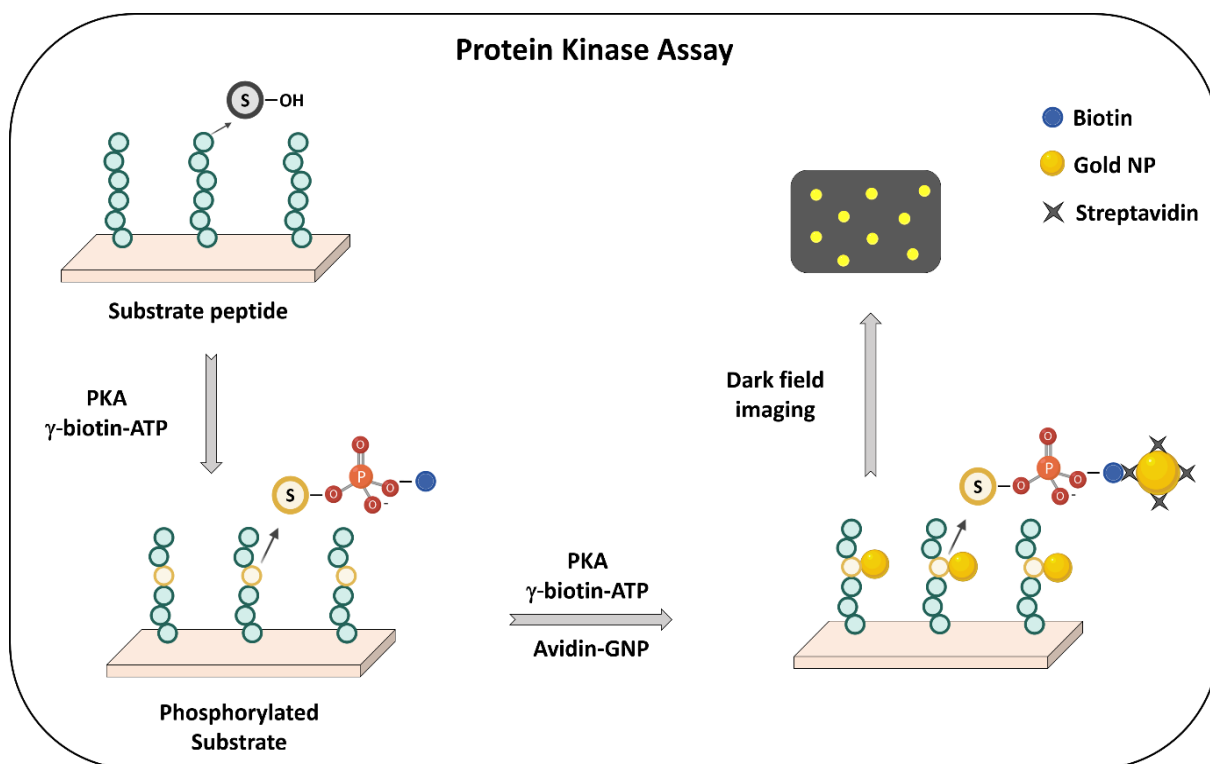


Figure 2. Schematic illustration of Protein Kinase Activity

5. Toxicity Associated with Nano Platforms

Nanotechnology in the recent past has gained wider attention and is being explored for delivering a number of therapeutic moieties. However, it is most important to address the nanoparticle related toxicity. NPs can enter the systemic circulation via several routes like topical, oral, inhalation, intravenous and others. Thus, the information obtained from the toxicity studies of the NPs serves as an essential data in determining the clinical applicability of NPs [56]. NPs having size less than 10 nm have proven to be acting similar to the gas molecules. They can enter the human tissues and can disturb the biochemical environment of the cell [57]. Several studies on animals and humans (clinical trials) have showed that the NPs once inhaled are distributed to heart, liver, spleen, lungs, brain and gastrointestinal tract [58–60]. To clear these NPs, the immune system and its components are activated in the body. The rough estimation of half-life of NPs is said to be about 700 days in lungs posing a serious threat to the respiratory system. Further, owing to their physicochemical aspects characteristic to different biological systems, NPs pose an unpredictable outcome on the human health. To bridge this gap, it is important to analyze the safety of nanoparticles being used for the delivery of the therapeutic cargoes.

6. Clinical Trials

The PKIs have been widely explored by the scientists all over the world and as a consequence, we have a number of drugs which have completed the clinical trials

and are approved by the regulatory bodies for the treatment of different types of cancer. In this context, we have summarized few of the recent on-going clinical trials in Table 2 to describe the target site of the therapeutic moieties along with its route of administration.

7. Conclusion

Protein kinase inhibitors represent a vast portion of novel therapeutic approaches in treating the diseases related to the dysregulation of protein kinases. Since their discovery, the focus on PKIs has been increasing enormously and several small molecules and antibodies have already been approved to be used as PKIs for cancer. As per the clinical research data, such targeted molecular drug along with conventional chemotherapy can provide the maximum therapeutic effect. Yet, reduced selectivity of these moieties and possibility of drug tolerance by the tumors, necessitate the development of novel strategies. Remarkably, the application of nanotechnology has provided a potential route for the delivery of therapeutic cargoes enhancing the therapeutic outcomes. This increase in efficacy is achieved by overcoming the limitations of solubility, bioavailability, targetability and possible adverse effects of the drug molecules. Recently, a number of therapeutic as well as imaging nanoplatfoms have been developed for the treatment of cancer via protein kinase inhibition. However, clinical applicability of such nanoplatfoms is limited due to several factors. Therefore, the upcoming studies should not only focus on developing the NPs for PKI delivery, but also evaluate its clinical applicability to benefit the human health against diseases like cancer.

Table 2. List of ongoing clinical trials of protein kinases inhibitors

Sl. No	Condition	Therapeutic moiety	Phase of clinical trial	Route of administration	Target site	Sponsor	Clinical trial gov. identifier
1	Kidney Cancer (Stage III & IV)	Lenvatinib, Pembrolizumab	Phase II & Interventional	Oral and IV	VEGFR2	Emory University	NCT04393350
2	Solid Tumors CNS Tumors	Entrectinib	Phase I & II	Oral	TRKA/B/C, ROS1, and ALK	Hoffmann-La Roche	NCT02650401
3	Lung Cancer	Gefitinib, EGFR816	Phase II	Oral	EGFR	Massachusetts General Hospital	NCT03292133
4	Colorectal Cancer	Camrelizumab, Apatinib	Phase II	IV	VEGF	Sun Yat-sen University	NCT04715633
5	Gastric Cancer	Apatinib	Phase II	Oral	VEGFR-2	Qinghai University	NCT03104283
6	Hairy Cell Leukemia	Ibrutinib	Phase II	Oral	BTK	National Cancer Institute (NCI)	NCT01841723
7	Papillary Thyroid Cancer	Imatinib	Phase I	Oral	PDGFR α	AHS Cancer Control Alberta	NCT03469011
8	High-grade Glioma	Sunitinib, Vandetanib, Erlotinib	NA	Oral	Multiple targets	VU University Medical Center	NCT02239952

Author Statement

All authors contributed equally to this work.

Conflict of Interest

The authors declare that they have no conflict of interest.

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