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Review Article

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Phosphatases of Regenerating Liver (PRL) as Therapeutic Targets in Cancer

Abstract

Background: Phosphatases of regenerating liver (PRL) represent a class of protein tyrosine phosphatases with oncogenic activity. PRL overexpression enhances cell proliferation, transformation, and promotes metastasis in many human cancers. Most notably, PRLs interact with a family of magnesium transporters, cyclin M (CNNM), to regulate intracellular Mg^{2+} levels. Thus, PRLs are attractive therapeutic targets given their role in oncogenic and tumour suppressor signaling pathways by modulating cellular growth.

Methods: Academic research articles were obtained by searching key terms in the PubMed database. This review specifically focuses on the articles that provided a comprehensive overview of PRLs, CNNMs, and small molecule inhibitors of PRLs.

Summary: This review discusses the role of PRLs in promoting cancer metastasis and explores current strategies for targeting PRL activity through the use of small molecule inhibitors. Although several potent PRL inhibitors have been discovered, improvements must be made prior to clinical applications. Therefore, understanding the molecular basis of PRL inhibition is essential for developing novel therapeutic agents in cancer treatments.

Introduction

Post-translational modification of proteins is a highly conserved process to install diverse functional groups onto synthesized proteins. One example of a reversible modification is phosphorylation, which involves the addition of a phosphate group onto the hydroxyl groups of a serine, threonine, or tyrosine residue.(1) In particular, protein tyrosine phosphorylation plays a fundamental role in physiological and pathological processes of eukaryotes.(2) Protein phosphorylation is implemented in regulating many signal transduction networks. Therefore, it is crucial to maintain a very precise balance of tyrosine phosphorylation and dephosphorylation in order to carry out proper cellular functions.(1) Two classes of enzymes exist to regulate the homeostasis of tyrosine phosphorylation: protein tyrosine kinases (PTKs), which catalyze tyrosine phosphorylation, and protein tyrosine phosphatases (PTPs), which are responsible for dephosphorylation.(1) PTPs are the largest family of phosphatases and are important modulators of signal transduction pathways.(2) PTPs are implicated in the control of cell growth, proliferation, differentiation, oncogenic transformation, and synaptic plasticity.(2) Disturbance in the balance between PTK and PTP activity results in abnormal tyrosine phosphorylation, which is linked to the development of many neoplastic diseases.(2) Phosphatases of regenerating liver (PRL) are a member of the PTP family that has implications in oncogenesis and cancer metastasis; thus, it is an attractive drug target for cancer therapies. The development of potent small molecule inhibitors to selectively target PRL activity suggests a promising approach for the treatment of many human cancers.(2)

PRL Phosphatases

PTPs are integral components of many signaling transduction cascades, such as the MAP kinase pathway.(2) The PTP family includes 107 members that can be subdivided into four classes.(3) Class I, II, and III PTPs utilize cysteine-based catalysis, whereas class IV utilizes aspartate-based catalysis.(3) Class I constitutes the largest and most diverse class, which includes tyrosine-specific classical PTPs containing receptor-like and non-transmembrane PTPs, and dual specificity phosphatases (DSPs).(3) DSPs are unique from the classical PTPs due to their ability to dephosphorylate both tyrosine and serine/threonine residues. Thus, DSPs tend to have the most diverse substrate specificity.(3) PRLs are classified as DSPs within the class I PTP family.(3) PRLs demonstrate oncogenic activity in which their overexpression promotes cell proliferation, transformation, and metastasis in several human cancers.(3) The PRL family is composed of three closely related members: PRL1, PRL2, and PRL3. PRL1 was the first member identified, and was initially characterized as a strongly upregulated immediate-early gene in the regenerating liver following partial hepatectomies.(4) All PRL proteins share high amino acid sequence similarity; PRL1 and PRL2 exhibit 87% similarity, while PRL3 exhibits 76% and 79% similarity to PRL1 and PRL2, respectively.(5) PRLs have low sequence homology with other DSPs (normally less than 30%), with the closest structural homology to VHR, Cdc14, and PTEN dual specificity phosphatases.(5)

Expression and Cellular Localization of PRLs

Although PRL family members possess similar sequences, the distribution of PRL proteins varies in eukaryotic cells. C.M. Dumanual et al. conducted an extensive analysis of PRL expression in human tissues. Among the three PRL phosphatases, PRL2 expression is the most abundant and is ubiquitously expressed in almost all tissues, while PRL1 appears to be more varied between tissue types.(6) Indeed, the widespread nature of PRL1 and PRL2 expression suggests their basic functions are common to many cell types. In contrast, PRL3 has a much more restricted expression pattern; it is primarily found in the heart and skeletal muscles, and is generally expressed at a lower level than PRL1 and PRL2.(6) Tissue specificity of PRL expression suggests non-overlapping functions for different PRLs. The highly controlled expression of PRL3 may explain why its overexpression is associated with the development of metastatic carcinomas.(7) In contrast, in mice, PRL1 and PRL3 expression levels are much more restricted, showing expression in the colon and intestine, but have very low or no expression in other organ systems.(8)

A unique feature of PRLs not found in other PTPs is the presence of a CAAX prenylation motif in the C-terminal domain preceded by a polybasic region.(9) The prenylation motif facilitates intracellular localization of proteins to the plasma membrane.(9) Furthermore, PRLs are associated with the plasma membrane and early endosomes in mammalian cells.(9) PRL proteins with a mutated or deleted CAAX sequence re-localize to the cytosolic or nuclear fraction.(9) The polybasic region adjacent to the prenylation site also promotes membrane localization of the PRLs, where complete substitution of six basic residues with alanine abolishes the plasma membrane association of PRL1. Additionally, membrane localization is likely controlled by the cell cycle, as all three PRLs have been suggested

to regulate cell cycle progression in mitosis.(10) This suggests that cellular localization of PRLs to the plasma membrane is highly correlated with their function in tumour metastasis.

PRL Structural Features

PRLs are approximately 20 kDa and are one of the smallest PTPs that consist of a single catalytic domain and lack a regulatory domain.(11) PRLs contain two sequence motifs central to the catalytic mechanisms utilized by the PTP family: the signature active-site motif $HC(X)_5R$ in the P-loop and the WPD loop motif.(12) The catalytic cysteine in the phosphatase motif carries out a nucleophilic attack upon phosphorylated substrates, resulting in the formation of a thiophosphoryl enzyme intermediate.(11) The microenvironment within the active site of PTPs causes the catalytic cysteine residue to have a particularly low pKa, around 5.(11) This allows the cysteine side chain to exist as a thiolate at physiological pH and to act as a nucleophile.(11) The essential role of the catalytic cysteine was confirmed by the C104A mutant, in which loss of the cysteine thiolate abolishes the catalytic activity of PRL3.(11) The P-loop also contains a conserved arginine involved in the stabilization of the transition state by providing a positive charge.(12) Correct positioning of this flexible P-loop was found to be critical for the activation and enzymatic catalysis in many PTPs.(12) Moreover, the neighbouring WPD loop in the active site plays a functional role in catalysis, as it contains a conserved aspartic acid which participates as the proton donor in both the formation and hydrolysis of the phosphoenzyme intermediate.(12)

PRL Phosphatase Activity

As a member of the PTP family, PRL activity occurs through a two-step catalytic mechanism, which involves the formation of a phosphoenzyme intermediate (Fig. 1). Previous studies have found low catalytic activity with PRLs. Specifically, wild-type PRL3 activity was three orders of magnitude lower than that of a typical DSP, such as CDC25.(13) Several differences between PRLs and classical PTPs may account for their low catalytic activity. Most notably, PRLs contain an alanine instead of the highly conserved serine/tyrosine residue next to the invariant arginine in the phosphatase motif.(11) The serine/tyrosine hydroxyl group normally functions in the breakdown of the phosphocysteine intermediate.(11) Consequently, this substitution in PRLs results in initial burst kinetics followed by slow turnover of the phosphoenzyme intermediate.(11) Mutagenesis studies confirm this catalytic mechanism, as the substitution of alanine to serine significantly improved both the burst kinetic rate and catalytic efficiency.(11) Similarly, the rate-limiting step of the catalytic mechanism has been identified as the hydrolysis of the phosphoenzyme intermediate, as it is extremely long-lived, with a half-life of over one hour.(11) G. Kozlov et al. show that this is partially responsible for the very low catalytic activity of PRL3, and suggests that the missing hydroxyl group may be provided by the substrate.(11) However, the physiological substrates of PRLs are still largely unknown due to the slow overall rate of catalysis.(11)

Regulation of PRL through Oxidation

The conservation of C49 in the active site of PRL phosphatases suggests that it has a functional role. There is growing interest in the redox of the catalytic cysteine in PTPs and its role in the regulation of signaling pathways in response to oxidative stress.(15) Recent data indicates two possible mechanisms for the oxidation of the catalytic cysteine side chain that involve its conversion to a sulfonic acid or the formation of an intramolecular disulfide bond.(16) PRL1 and PRL3 are capable of forming an intramolecular disulfide between C49 and the catalytic cysteine C104.(17) In PRL2, this bond forms at analogous positions between C46 and C101.(17) The formation of the redox-dependent disulfide bond results in the loss of the catalytic cysteine thiolate, which blocks substrate binding and catalysis.(11) Since PRLs are prone to oxidation, this feature may also contribute to the low dephosphorylation activity observed.(11) Studies have shown that PRL1 can be oxidized *in vivo* by H_2O_2 treatment, where its redox status is controlled by the cellular glutathione system.(17) Several other DSPs are also subject to redox regulation, such as PTEN, CDC25, and MKP formation of disulfide bonds following H_2O_2 treatment.(18) Thus, the disulfide bond between C49 and catalytic cysteine C104 in PRLs is thought to protect the catalytic cysteine from irreversible oxidation during oxidative stress.(17)

PRL Interaction with Cyclin M (CNNM) Magnesium Transporters

PRL have been identified as oncogenes, yet no physiological substrate has been identified due to their slow catalytic rate.(11) However, recent studies demonstrate PRLs can bind to cyclin M (CNNM) family proteins.(19) This interaction is independent of their phosphatase activity and indicates a potential oncogenic mechanism through magnesium homeostasis regulation.(19)

The CNNM family proteins, also known as ancient conserved domain proteins, are transmembrane proteins involved in mediating Mg^{2+} efflux in mammalian cells.(20) The CNNM family is composed of four members, CNNM 1-4.(20) CNNM2 and CNNM3 are ubiquitously expressed in mouse tissue, CNNM1 is mainly in the brain, and CNNM4 is within the gastrointestinal tract.(21) CNNMs demonstrate significant sequence homology to other Mg^{2+} membrane transporters.(22) CNNMs contain an extracellular region, transmembrane domain, conserved cystathionine-B synthase (CBS) pair domain, and putative cyclic nucleotide binding domain.(22) In particular, the CBS-pair domain is conserved within other bacterial Mg^{2+} membrane transporters, such as CorC and MgtE.(22) The CNNM CBS-pair domain forms a dimer and is generally involved in nucleotide binding, particularly to adenylate nucleotides.(22) The Mg^{2+}-ATP complex induces a conformation change in CBS-pair domain dimers to regulate protein function.(22) CNNM transporters play a significant role in maintaining intracellular Mg^{2+} homeostasis for proper cellular functions.(23) Mg^{2+} is the most abundant divalent cation and is required as a cofactor for many enzymes involved in energy metabolism and genomic stability.(23) Moreover, increased intracellular Mg^{2+} is associated with increased cell proliferation.(23) CNNM-dependent Mg^{2+} efflux suppresses tumour progression by regulating energy metabolism and AMPK/mTOR signaling.(23)

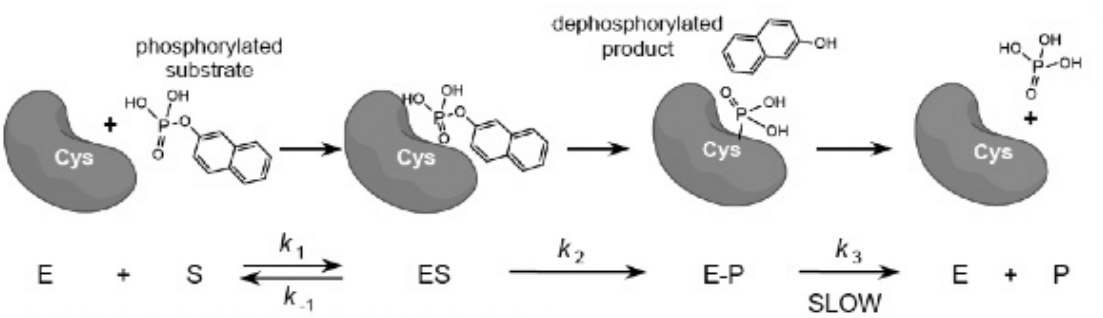

Fig. 1. Catalytic cycle of PRL phosphatases. PRL catalytic mechanisms occur through two steps. Initial burst kinetics is followed by a long-lived phosphocysteine intermediate. Hydrolysis of the intermediate is the rate-limiting step. (Fig. from Gulerez *et al.*(14))

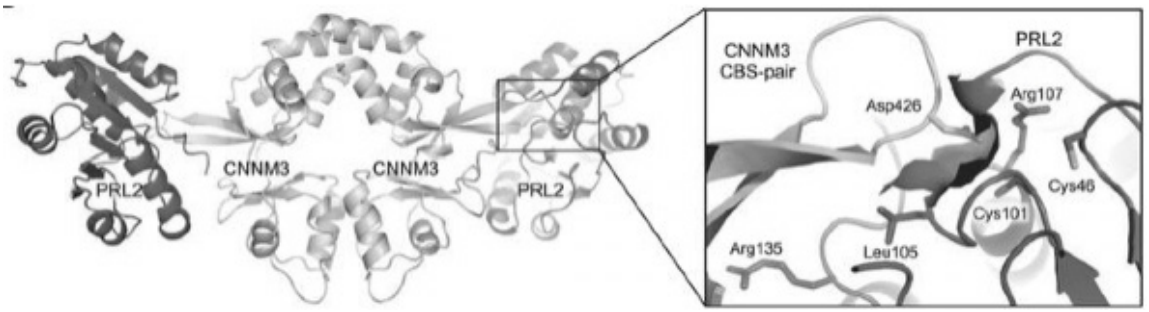

Fig. 2. Complex of PRL2 with the CBS-pair domain of CNNM3. CNNMs form a central homodimer that binds PRLs via an extended loop. A CNNM aspartic acid residue inserts into the phosphatase active site and is essential for complex formation. The catalytic cysteine contacts the CNNM aspartic acid to close the substrate pocket. (Fig. from Gulerez *et al.*(14))

PRLs interact with CNNM transporters to regulate Mg^{2+} transport.(14) CNNM and PRL complex formation is mediated by an extended loop in the CBS-pair domain, which contacts the PRL active site.(14) Mutagenesis studies by Gulerez et al. demonstrate a key D426 residue in CNNM3 that plays an important role in binding (Fig. 2).(14) The aspartate residue is inserted into the catalytic pocket and likely mimics the negatively charged phosphate group of a bound substrate. Therefore, PRLs act as pseudo-phosphatases, which are specific for CNNM proteins.(14) Additionally, the PRL-CNNM interaction is negatively regulated by the phosphorylation and oxidative state of PRL catalytic cysteine.(14) The phosphocysteine intermediate blocks CNNM binding through steric and electrostatic repulsion of D426.(14) The strong association between PRLs and metastatic diseases may be explained by its interaction with CNNM tumour suppressors to disrupt Mg^{2+} homeostasis.(23) PRL phosphatases are endogenously cysteine-phosphorylated in response to intracellular Mg^{2+} levels.(14) Through direct interactions, PRL binding inhibits CNNM-dependent Mg^{2+} efflux to raise intracellular Mg^{2+} levels to promote proliferation.(14) This supports the observation that Mg^{2+} deprivation leads to decreased PRL phosphorylation and increased total PRL levels, which suggests that the oncogenic property of PRLs is likely dependent on its interaction with CNNM Mg^{2+} transporters.

CNNM-PRL interaction is also regulated by PRL active site oxidation. (14) Oxidation of the PRL catalytic cysteine leads to decreased CNNM CBS-pair domain binding affinity.(14) Oxidation of the catalytic cysteine to form a disulfide bond induces an active site conformation change that inhibits CNNM binding (Fig. 3).(14) Catalytic cysteine phosphorylation may also be controlled by oxidation of the catalytic site, as PRL phosphorylation can only occur in the fully reduced form.(14)

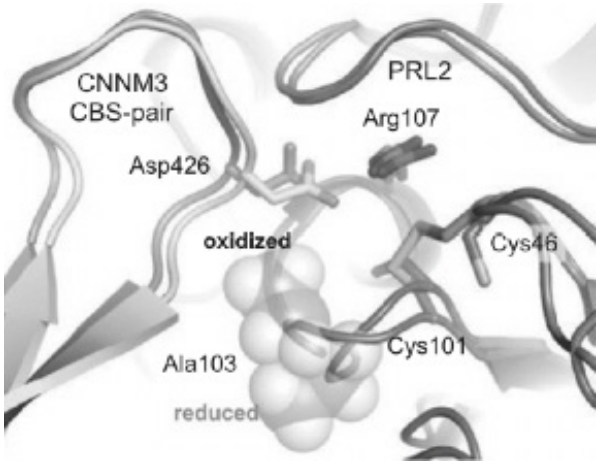

Fig. 3. Structural change of PRLs upon oxidation. A conformational change can be observed between the reduced and oxidized PRL2 complexes. Upon oxidation, the catalytic cysteine, Cys101, moves away from the CNNM3 Asp426 and Ala103 flips to displace CNNM3. (Fig. from Gulerez *et al.*(14))

Role of PRLs in Cancer

PRLs were first identified as a potential oncogene in 2001 through gene expression profiling.(7) S. Saha et al. discovered that PRL3 mRNA was consistently elevated in all metastatic lesions derived from colourectal cancer, whereas minimal PRL expression was observed in non-metastatic samples.(7) Aberrant elevation of PRL3 expression was also found in breast, lung, cervical, ovarian, and gastric cancers.(7) PRL3 expression is correlated with disease progression in ovarian cancers, with elevated levels in advanced stages.(24) Finally, increased tumour invasiveness is commonly observed in breast cancer patients expressing high levels of PRL3, suggesting that PRL3 expression can be used as a prognostic factor to predict advanced stages of disease.(25)

Enhanced PRL expression in cells results in increased cell adhesion, migration, invasiveness, and proliferation. Q. Zeng et al. demonstrated that PRL1 and PRL3 overexpression promotes cell mobility, cell invasiveness, and metastasis in Chinese hamster ovary cells, whereby catalytically inactive PRL3 reduced the ability to promote migration.(26) Y. Wang et al. also revealed that PRL2 can affect cell migration and invasion with human lung cancer cells.(27) PRL2 knockdown by short hairpin RNA significantly reduced tumour cell migration and invasion.(27) Both studies indicate that the oncogenic effect of PRLs is dependent on its phosphatase activity, as PRLs with mutated catalytic cysteine and arginine lose the ability to promote cell migration and metastasis.(27)

Furthermore, PRLs have been shown to affect cell apoptosis and angiogenesis. Recent studies reveal a novel connection between PRLs and p53 tumour suppressor.(28) S. Basak et al. demonstrated that, upon DNA damage, upregulation of PRL3 occurred in a p53-dependent manner to induce cell cycle arrest.(28) This mechanism occurs through increased Akt activation, negative feedback of the PI3K/Akt pathway, and transcription of growth arrest genes.(28) Similarly, p19Arf was upregulated in PRL3 knockout cells leading to MDM2 sequestration and p53-dependent cell cycle arrest.(28) PRL3 has also been found to facilitate angiogenesis, as it is strongly expressed in tumour vasculature.(29) PRL3 is involved in triggering tumour angiogenesis by downregulation of interleukin-4 to attenuate its inhibitory effect on vasculature formation.(29)

PRL Regulation of Signaling Pathways

PRL3 has been shown to alter several major oncogenic and tumour suppressor cell signal transduction pathways, including PTEN, p53, and Src pathways.(30) Notably, PRL3 appears to activate Src and PI3K/Akt signaling by reducing the expression of a negative regulator, Csk and PTEN respectively, to promote cellular growth, proliferation, and survival.(30) F. Liang et al. showed that PRL3 overexpression in HEK293 cells results in Csk downregulation and increased Src kinase activity.(31) Src pathway activation increases phosphorylation of downstream targets ERK1/2, STAT3, and p130CAS, leading to increased cell mobility and growth.(31) Specifically, ERK1/2 kinase activation contributes to a sustained G1 to S phase of cell cycle progression and proliferation.(31)

PRL interaction with adhesive proteins, such as cadherin and integrin, are also involved in modulating cell migration and invasiveness.(32) PRLs can interact with integrin proteins to enhance the binding to Src kinases to activate downstream signaling pathways.(33) Furthermore, E-cadherin and vinculin are downregulated with PRL3 overexpression, resulting in PI3K/Akt pathway activation by PTEN downregulation, a negative regulator of PI3K.(32) PRL-mediated signaling can also be explained by the activation of an extensive signaling network via receptor tyrosine kinases (RTKs). PRL3 regulates epidermal growth factor receptor (EGFR) by transcriptionally downregulating PTP1b, resulting in EGFR hyperphosphorylation and activation, which promotes growth.(34) Altogether, these studies strongly suggest that PRL3 overexpression is linked to the development of metastatic cancers by activating signaling pathways to enhance cellular proliferation. Therefore, PRL3 makes a very attractive target for small molecule inhibitors to halt the advancement of tumour progression.

Targeting PRLs using Small Molecule Inhibitors

Given that PRL overexpression is strongly associated with tumour progression and metastasis, there is increasing interest in discovering novel therapeutic agents to target the oncogenic properties of PRLs. Several small molecule inhibitors have been reported to inhibit PRL activity. For example, pentamidine was discovered to inhibit the activity of several PTPs, including PRL *in vitro*.(35) Pentamidine treatment is effective at inhibiting tumour growth induced by human melanoma cells.(35) However, pentamidine demonstrates nonspecific inhibition of all three PRLs, as well as an inhibitory effect on several other PTPs, such as PTP1b and MAPK phosphatase. Therefore, it cannot be concluded that the effect on tumour growth was solely due to PRL inhibition.(35)

More recently, high-throughput screening of the Roche chemical library revealed rhodanine derivatives and thienopyridone inhibit PRL3 activity.(36) Specifically, benzylidene rhodanine derivatives demonstrate greater potency of PRL3 inhibition than pentamidine, and is also more effective in decreasing the invasiveness of mouse melanoma cells.(36) The most noteworthy compound identified from the Roche chemical library was thienopyridone (7-amino-2-phenyl-5H-thieno-[3,2-c]pyridine-4-one), which exhibits an IC_{50} value of 132 nM against PRL3.(37) It displays a very high selectivity towards PRLs with minimal activity against 11 other phosphatase *in vitro*.(37) Furthermore, thienopyridone significantly inhibits tumour cell anchorage dependent growth of colon cancer cells and suppresses cell migration through p130Cas cleavage induction.(37) However, the main concern with thienopyridone is its high electron density, which may potentially cause idiosyncratic drug toxicity.(38) An improved inhibitor, iminothienopyridinedione (7-iminothieno-[3,2-c]pyridine-4,6(5H,7H)-dione), can be derived from thienopyridone through photooxygenation (Fig. 4).(38) It exhibits a greatly improved IC_{50} of 18 nM against PRL3, making it the most potent PRL3 inhibitor reported to date.(37) One advantage of iminothienopyridinedione is its decreased electron density and potential redox liability.(38) It is also 10-fold more potent than thienopyridone and has greater stability in solution.(38) These properties are important in decreasing off-target toxicity for improved drug absorbance and pharmacological response.(38)

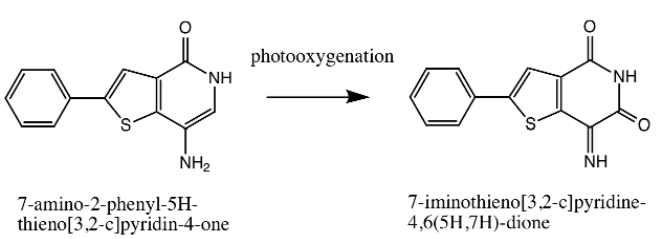

Fig. 4. Iminothienopyridinedione produced from thienopyridone through photooxygenation. The improved inhibitor iminothienopyridinedione has a 10-fold potency compared to thienopyridone. (Fig. from Salamoun *et al.*(38))

Conclusion

Phosphatases of regenerating liver are protein tyrosine phosphatases that play a critical role in cancer progression and tumour metastasis by inducing cell proliferation, survival, migration, and invasion. PRLs associate with CNNM magnesium transporters to promote oncogenic transformation through the regulation of intracellular magnesium levels. Evidence indicates that PRLs affect many important oncogenic and tumourigenic pathways, thus making it an attractive therapeutic target for cancer treatments. Several potent and selective PRL inhibitors, such as thienopyridone, have been discovered as promising anti-cancer agents. Although it is known that the oncogenic property of PRLs is dependent on its phosphatase activity, the mechanism in which small molecule inhibitors decrease PRL activity is still unknown and remains a compelling area of research. Determining the molecular basis of PRL inhibition is essential for the development of highly selective PRL inhibitors with improved pharmacological properties.

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