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PROTEIN KINASES AND PHOSPHATASES AS DRUG TARGETS TO CURE HUMAN MALIGNANCIES

PROTEINE CHINASI E FOSFATASI: BERSAGLI TERAPEUTICI PER IL TRATTAMENTO DI NEOPLASIE UMANE

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Abstract.
Protein phosphorylation controls various aspects of cell proliferation, differentiation, metabolism, survival and motility. Protein kinases and phosphatases are involved in intracellular signal transduction pathways providing a dynamic and reversible regulation of many biological activities. One of the biggest challenges of modern medicine is to identify promising therapeutic targets among these families of enzymes, which are involved in the development of various human cancers.
This review focuses on protein kinases and phosphatases as targets of small-molecule drugs. Some examples of breakthrough compounds are highlighted to illustrate achievements and challenges in the generation of small-molecule inhibitors to cure human malignancies.

KEYWORDS: phosphorylation, protein kinases, protein phosphatases, cellular signal transduction, drug targets, small-molecule inhibitors.

Riassunto.
La fosforilazione proteica regola diversi aspetti della sopravvivenza, della proliferazione, della differenziazione, del metabolismo e della motilità della cellula. Le proteine chinasi e fosfatasi sono coinvolte nella propagazione dei segnali intracellulari, creando una regolazione dinamica e reversibile di molte attività biologiche. Una grande sfida della medicina moderna consiste nell'identificare bersagli terapeutici tra questi enzimi, legati all'insorgere di varie neoplasie umane.
Questa review descrive alcune chinasi e fosfatasi identificate come bersagli terapeutici di inibitori a basso peso molecolare. Alcuni esempi di molecole rivoluzionarie evidenziano i successi e le difficoltà nella creazione di inibitori a basso peso molecolare per curare neoplasie umane.

PAROLE CHIAVE: fosforilazione, proteine chinasi, proteine fosfatasi, vie cellulari di signalizzazione, bersagli terapeutici, inibitori a basso peso molecolare.

Introduction

Protein phosphorylation regulates almost all aspects of the life of the cell. This process comprises the transfer of a phosphate group from a donor molecule (usually ATP) to serine, threonine or tyrosine residues in an acceptor protein.
Already in the 19th century it was known that phosphates could be bound to proteins. Most examples of these 'phosphoproteins' were found in milk (caseins) and egg yolk (phosvitin) and were simply considered a biological method of providing phosphorus as a nutrient. Therefore, the existence of phosphoproteins was considered a consequence of metabolic reactions, and nothing more, for almost 100

years after their discovery (1).
In the 1950's this all began to change as phosphoproteins began to emerge as key regulators of cellular life. In 1954, an enzyme activity was observed that transferred a phosphate onto another protein (2), a reaction called phosphorylation. The protein responsible was a liver enzyme that catalyzed the phosphorylation of casein and became known as a protein kinase, the first of its kind to be discovered. A year later, the role of phosphorylation became more interesting as Fischer and Krebs (3), followed by Wosilait and Sutherland (4), showed that an enzyme involved in glycogen metabolism was regulated by the addition or removal of a phosphate, suggesting that reversible phosphorylation could control enzyme activity. This idea was later proven to be true and has now seeped into virtually every aspect of cell biology.
Today, it is thought that one third of the proteins present in a typical mammalian cell are covalently bound to phosphate (meaning that they are phosphorylated at one time or another). Cell biology provides us with many examples of regulation by phosphorylation: increasing or decreasing the biological activity of an enzyme, helping move proteins between subcellular compartments, allowing interactions between proteins to occur, as well as labelling proteins for degradation. Targets of phophorylation are enzymes, structural proteins, cell receptors, ion channels and signalling molecules. The variety is immense and now many human diseases have been recognized to be associated with the abnormal phosphorylation of cellular proteins. These developments have brought the study of phosphorylation into the central stage of medical research, a fact that was recognized in 1992 when Fischer and Krebs received the Nobel Prize in medicine for their pioneering efforts (5).

Phosphorylation as a biological mechanism to transduce signals

How can phosphorylation control enzyme activity? Phosphorylation refers to the addition of a phosphate to one of the amino acid side chains of a protein. Proteins are composed of amino acids bound together and that each amino acid contains a particular side chain, which distinguishes it from other amino acids. Phosphates are negatively charged (with each phosphate group carrying two negative charges) so that their addition to a protein will change the characteristics of the protein. This change is often a conformational one, causing the protein to change how it is structured (Fig. 1).
This reaction is reversible by a process called dephosphorylation. The protein switches back to its original conformation when the phosphorus is removed (Fig. 1). If these two conformations provide the protein with different activities (i.e. being enzymatically active in one conformation but not the other), phosphorylation of the protein will act as a molecular switch, turning the activity on or off. The transfer of phosphates onto proteins is catalyzed by a variety of enzymes in the cell. Although the variety is

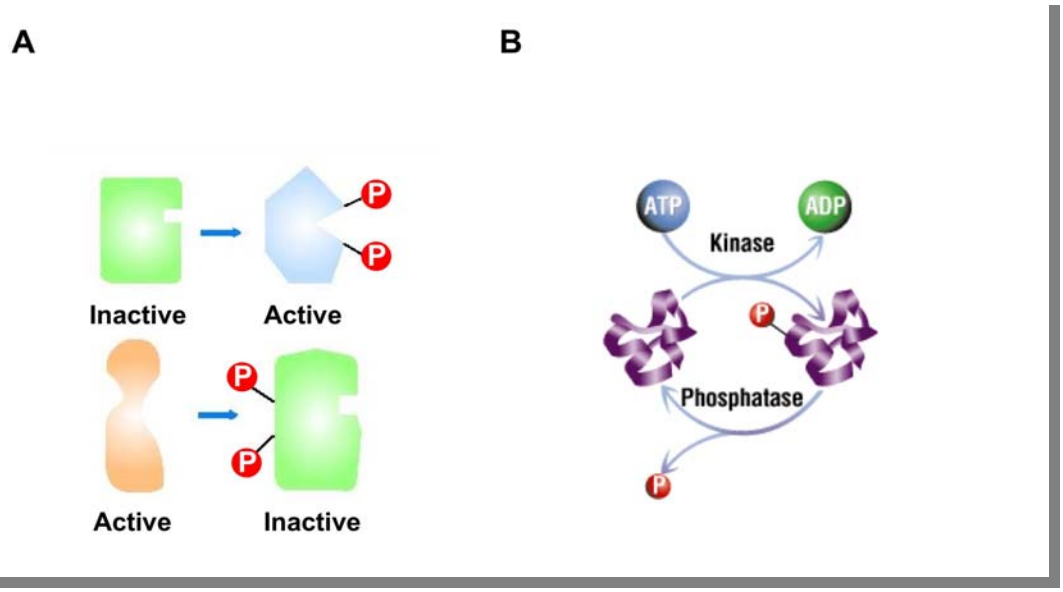

Fig. 1: Mechanisms of protein phosphorylation. A. Phosphorylation reactions often induces conformational changes in proteins affecting their activities, either enhancing it (upper panel) or decreasing it (lower panel). B. Kinase and phosphatase activities. The addition of a phosphate group to an acceptor protein is catalyzed by protein kinases to the expenses of an ATP donor molecule. The inverse reaction is catalyzed by protein phosphatases families of proteins.

large, all of these enzymes share certain characteristics and fall into one class of proteins, called protein kinases (6). There are 518 protein kinases in humans that have been classified in 20 different families based on amino acid sequence comparisons (7) (Fig. 2). A second class of enzymes is responsible for the reverse reaction, in which phosphates are removed from a protein. These are termed protein phosphatases. Phosphatases are structurally and functionally diverse enzymes that are represented by three distinct gene families. Within each family, the catalytic domains are highly conserved, with functional diversity endowed by regulatory domains and subunits. There are almost 100 genes predicted to encode protein phosphatases in humans, which is about a quarter the number of protein kinases implying a higher degree of redundancy and substrate promiscuity among phosphatases (8). In addition, a number of signal transduction events are regulated by non-protein kinases like the lipid kinase family. Similar to protein kinases also lipid kinase activity is reversed by lipid phosphatases. One of the best studied pathways is controlled by various phosphorylated forms of

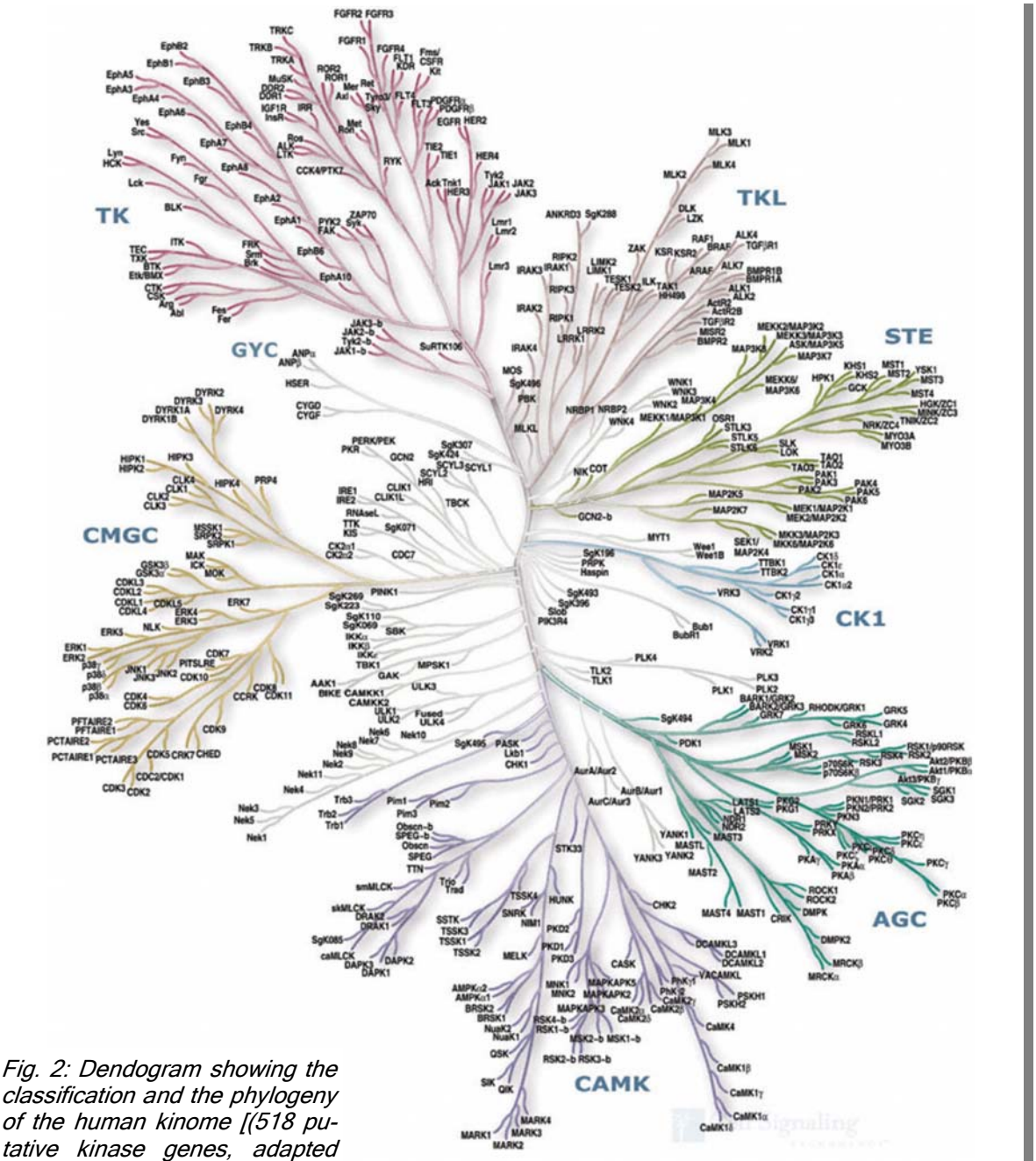

Fig. 2: Dendogram showing the classification and the phylogeny of the human kinome [(518 putative kinase genes, adapted from (Manning G et al., Science 2002)].

phosphatidylinositol that can undergo reversible phosphorylation at multiple sites and plays a crucial role in cell proliferation, differentiation, apoptosis, cytoskeletal reorganization, membrane trafficking as well as in the regulation of protein synthesis and metabolism. The use of the phosphorylation/dephosphorylation of a protein as a control mechanism has many advantages: (i) it is rapid, taking as little as a few seconds; (ii) it does not require new proteins to be made or degraded; (iii) it is easily reversible. The extensive use of this control mechanism is apparent by the large number of known kinases and phosphatases (6).

Importantly, phosporylation reactions are fundamental to transduce external cues to the inside of the cell. The reception of a signal on the surface of a cell often results in the activation of kinases and phosphatases (6). Once activated, cellular phosphorylation patterns will begin to change, with various proteins being phosphorylated or dephosphorylated. The final result will be various changes in cellular behaviour. Many of the proteins that are phosphorylated upon reception of a signal are protein kinases as well. This organization of kinases produces a phosphorylation cascade (6), in which one protein kinase is activated by phosphorylation upon reception of a signal; this kinase then phosphorylates the next kinase in the cascade, and so on until the signal is transmitted through the cell. In such a system, the kinase cascade can start with the receptor itself (which is often a kinase) or a free-floating cytoplasmic kinase. Upon reception of a signal, these phosphorylation cascades continue to function until protein phosphatases are activated and shut off their transmission. In animal cells, these cascades are mediated by two types of kinases: serine/threonine kinases (6) (which phosphorylate serine and threonine amino acid side chains) and tyrosine kinases (6) (which phosphorylate tyrosine amino acid side chains).

The central role of phosphorylation dependent signalling in the control of a large number of cellular processes make kinases and phosphatases very attractive drug targets, which is the subject of this review. As a consequence, detailed knowledge of the structure of kinases and phosphatases is of pivotal importance for any rational drug design approach. In particular kinases have been selected as targets for therapeutic invention since about 80 kinases map to loci that are implicated in human diseases and 164 kinases map to amplicons that have been detected frequently in tumours. A large number of kinase inhibitors entered clinical trials as anti cancer drugs, since these molecules have the potential of being less toxic and more specific than currently used generic chemotherapeutic agents. Kinases play also a major role in other disease areas like immunosuppression, arthritis, neurodegeneration, retinopathy, schizophrenia, hypertension and obesity. In this review, far from wanting to cover in a comprehensive manner the extensive literature on the subject, I will provide only some paradigmatic examples, which show the great therapeutic potential of small molecules inhibiting protein kinases and phosphatases activities to cure human cancers.

Protein kinases as cancer therapeutic targets

Protein kinases can be simplistically subdivided in four groups: 1) growth factor receptor tyrosine kinases (EGFR family, VEGFR family, PDGFR, KIT, FGFR, IGFR etc.), 2) non-receptor tyrosine kinases (Abl, Src family, FAK1), 3) growth factor receptor serine/threonine kinases (e.g. TGFβR) and 4) non-receptor serine/threonine (e.g. MAPK family, CDK family, Raf kinase, PI-3K, PKC, PKB/Akt, mTOR and aurora kinase). The role of protein kinases in several pathogenic processes, such as oncogenic transformation, has been known for 30 years. However, in the beginning they were dismissed as targets for therapy due to the lack of knowledge on their specific roles and the concerns about drug safety. In more recent years, the fear left the place to the excitement when safe and effective small molecules inhibiting specific protein kinases have been discovered and developed. Here I will discuss some proof-of-concept examples of inhibitors for three of the four groups of protein kinases, which I listed above:

1) The epidermal growth factor receptor (EGFR) family have key roles in fetal development, tissue repair, and disease processes such as neoplastic formation (9). In normal cells EGFR tyrosine kinase (TK) activity is tightly regulated at multiple levels, influenced by factors such as availability of ligand and degradation of the receptor by intracellular endocytosis (10). Many human tumours have increased or altered expression of EGFR-TK and its ligands (11). Expression of EGFR and its ligands is correlated with patient outcomes in a number of solid cancers, such as head and neck squamous cell carcinoma (12) and lung adenocarcinoma (13,14). Because of the importance of EGFR-TK activation in transduction of growth signals in tumours, considerable effort has been put into developing cancer treatment approaches that target EGRF-TK. A successful approach has been to target the EGFR tyrosine kinase activity with small molecule inhibitors which compete with adenosine triphosphate (ATP) for binding to the ATP-binding pocket of EGFR-TK, located inside the cell (15).

Different inhibitors were derived from a combination of molecular modelling and random screening of libraries of libraries of small compounds; molecular modelling was based on x-ray crystallographic structures of EGFR-TK as well as on the structures of natural tyrosine kinase inhibitors such as soy-bean derived genistein (15). Among several inhibitors developed with these approaches, the main stage is taken by Gefitinib (commercialized with the name Iressa® by AstraZeneca). Gefitinib can rapidly disable signal transduction pathways downstream of EGFR (16,17). Gefitinib treatment in preclinical tumours has resulted in additive or synergistic tumour inhibition when combined with chemotherapy agents or radiation therapy (18,19). Therefore, anti-EGFR-TK agents have clearly the potential to treat common solid cancers.

2) In chronic myelogenous leukaemia (CML), a balanced reciprocal chromosomical translocation in hematopoietic stem cells (HSCs) produces the hybrid BCR-ABL gene, which encodes the oncogenic Bcr-Abl fusion protein. In normal conditions, the ABL gene produces a tightly regulated non-receptor protein tyrosine kinase (Abl). Abl plays a fundamental role in regulating cell proliferation, adherence and apoptosis (20). In contrast, the BCR-ABL fusion protein encodes a constitutively activated kinase. This event induces HSCs to have a deregulated clonal proliferation, decreased ability to adhere to the bone marrow stroma and a decreased apoptotic response to mutagenic stimuli, which triggers with time malignant transformations. The resulting granulocytes fail to develop into mature lymphocytes leading also to an increased susceptibility to infection. This aetiology renders Bcr-Abl protein kinase an attractive target for drug intervention in CML. Here again, as in the case of EGFR-TK, a successful strategy has been to target the ATP-binding site of Abl and to screen large and diverse compound archives for the capacity to inhibit Abl tyrosine kinase activity. This was done at Novartis Pharma (Basel, Switzerland) and led to the identification of pyrimidine A as a structurally attractive lead molecule to target Abl (21). Chemical modifications of this lead molecule, driven by several structure/activity studies, led to the development of Imatinib (commercialized as Glivec®). Glivec is well tolerated in CML patients, with nearly all the subjects in chronic phase showing a complete haematological response and half of them showing also a cytogenic response (22). The key of the success of Glivec® has been to precisely target the underlying cause of CML, that is to inhibit with efficacy the mutant protein Bcr-Abl with a molecule tuned chemically to have an excellent inhibitory and a safe toxicological profile.

3) mTOR is a serine/threonine kinase that integrates nutrient and mitogenic stimuli to regulate cell growth and proliferation (23). mTOR is under direct influence of PI-3K-PKB/Akt pathway, since it can be phosphorylated, both in vitro and in vivo, by PKB/Akt on Ser2448, suggesting that PKB/Akt may directly modulate the function of mTOR (24,25). In the cell, mTOR enhances phosphorylation of S6K1 and the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), which upregulates translation of several specific mRNAs and increases available translation initation factor 4E (eIF4E) (26) (Fig. 3). mTOR pathway is fundamental in normal progression of the cell cycle, since both the downstream S6K1 and the 4E-BP1/eIF4E pathways are required for and independently mediate mTOR-dependent G1 phase progression (27) (Fig. 3). Thus, it is not astonishing that deregulation in mTOR pathway is frequently observed in several human malignancies (28,29). In this case, a useful hint to search and discover new therapeutic mTOR inhibitors came by Nature: mTOR is inhibited by rapamycin, a macrolide initially identified as an antifungal. Rapamycin binds avidly to the immunophilin FK506 binding protein 12 (FKB12), and the resultant complex inhibits the phosphorylation of mTOR downstream targets, ultimately leading to cell cycle arrest (30,31). The tumour-growth-inhibitory properties of rapamycin were early recognized (32); however, initially rapamycin was clinically developed and currently used for its immunosuppressive properties. Moreover, rapamycin has an undesirable pharmacological profile, including the fact that it is poorly soluble in water and unstable. Therefore, based on rapamycin

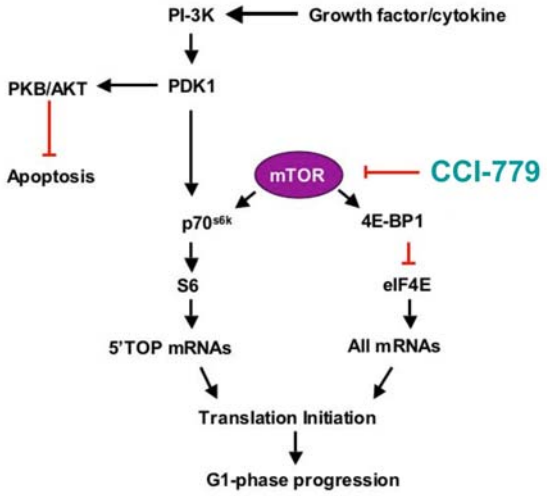

Fig. 3: Schematic representation of mTOR signalling pathway and site of pharmacological action of rapamycin analog CCI-779 (in green). See text for detailed description.

structure, pharmaceutical companies invested considerable effort to develop rapamycin analogs, which are now in or near clinical trials. The reader is referred to a recent review to find an exhaustive description of rapamycin analogs for clinical use (33), while here I will focus only on one of them, which is the promising cell cycle inhibitor 779 (CCI-779) (Fig. 3). CCI-979 formula was developed by Wieth-Lederle (USA): CCI-779 has been shown to have antitumor effects in animal studies (34,35). A number of clinical trials have been completed with CCI-779 in patients with different malignancies, such as glioblastoma, melanoma, glioma, mantle-cell non-Hodgkin's lymphoma, small cell lung cancer, and renal cell carcinoma (33,36). Overall, the results of these trials showed that CCI-779 exhibit a promising clinical activity in a significant sub-population of cancer patients. Current efforts in the private sector are aimed to understand in which precise patient population this agent is more effective and if its activity could be more optimal if combined with other therapeutics (chemotherapy, radiotherapy etc.).

Protein phosphatases as cancer therapeutic targets

Protein phosphatases may be considered promising targets for small molecule-based inhibiting strategies in some cancers, although less is known and the investment of resources by the industry is much less consistent, compared to their biological counterpart, protein kinases. Protein phosphatases can be diversified in protein phosphatases (either on tyrosine or serine/threonine residues), lipid phosphatases and dual activity phosphatases (displaying both protein and lipid phosphatases activities), according to their substrate specificities. A good example of successfully 'druggable' phosphatases to treat cancer is the family of PTPs Cdc25s, composed by three members, which activate cyclin-dependent kinases (37). Among these, Cdc25B is highly overexpressed in more than 30% of human primary breast cancers. For this reason, several pharmaceutical companies are now developing small molecule Cdc25B inhibitors, which have been proven to be effective in tumour models (38), to enter them in human clinical trials.

An other candidate phosphatase to be targeted to cure cancer is Prl-3. Prl-3 is specifically overexpressed in metastasis but not in the originating primary tumours (39), strongly suggesting that it may be essential for the formation of metastases but not for normal tissue growth. If these findings will be confirmed in the future, Prl-3 may constitute a promising small-molecule drug target to inhibit metastasis formation in human malignancies. Interestingly, a recent study on *in vitro* cellular models uncovered the mechanism of action of Prl-3 in promoting cancer cell invasion (40): this occurs through downregulation of PTEN and upregulation of the PI-3K/PtdIns(3,4,5)P3 pathway, described further below in this section.

In the cell, signalling systems are exploited for multiple and interconnected functions in physiological and pathological states, and strategies aiming at inhibiting phosphatase-dependent signalling pathways with small molecules should be carefully experimentally proven, in order to do not impact negatively 'healthy' homeostasis. An established paradigm of this concept is the PI-3K/PtdIns(3,4,5)P3 signalling, mentioned above. One phosphatase that seems to be important in attenuating PI-3K/PtdIns(3,4,5)P3 signalling, upregulated in many malignancies, is the lipid phosphatase known as phosphatase and tensin homologue on chromosome 10 (PTEN). PTEN is a major regulator of PI3K signalling in many cell types, and functions as a tumour suppressor due to antagonism of the anti-apoptotic, proliferative and hypertrophic activities of the PI3K pathway (41). In addition, in the last five years multiple in *vitro* and *in vivo* studies demonstrated the fundamental role that PTEN plays also in insulin signal transduction in target tissues (42,43). Tissue-specific PTEN knock-out mice (in adipose tissue, muscle and liver) display improved glucose tolerance, thus ameliorating systemic insulin sensitivity and reversing diabetes in these animal models (42,43). However, liver specific PTEN knock-out or decrease in its expression in the long-term may favour the development of liver steatosis and hepatocellular carcinoma (44-46). Therefore, even if PTEN has been proposed as a useful small molecule drug target to treat diabetes/insulin resistance (47), this could be a double-edge sword and serious concerns exist for the safety and effectiveness of this strategy in humans.

Transient protein-protein interactions as cancer therapeutic targets

Since dysregulation in the cell cycle is an hallmark of all cancers, protein kinases and phosphatases that control the cell cycle could be interesting anticancer targets (48). As already mentioned in the section on phosphatases, Cdc25 phosphatases are particularly attractive candidates for the development of cancer therapeutics because of their role in promoting cell-cycle progression and their observed overexpression in many cancers (49). During cell proliferation, Cdc25 phosphatases dephosphorylate and thereby activate the cyclin-dependent kinase (CDK)-cyclin complexes.

One of the most recent and innovative strate-

gies to cure human malignancies is to identify drugs that target transient protein-protein interactions as a therapeutic target. In fact, the interactions of protein kinases and protein phosphatases with their protein substrates are transient, and are therefore poorly characterized and problematic for drug discovery. On the other hand, the best inhibitors discovered so far protein-protein interactions have been described for stable, not transient, protein complexes (50). Currently there are no available valid inhibitors for transient kinase/phosphatase interactions with their substrates exploited in the clinics, but advances in the technologies and in the knowledge could reasonably soon lead to the identification of such inhibitors. A paradigm of this concept is the transient interaction between Cdc25 and phosphorylated CDK-cyclins. A docking site remote from the active site in Cdc25 that mediates efficient recognition of phosphorylated CDK-cyclins has been identified (51). Crystallographic (52,53), structural (site-directed mutagenesis) (51,54,55) and computational (56,57) studies validated a model of the docking orientation of CDC25B with its CDK–cyclin substrate in which the interfacial contacts in the remote docking site are primarily ionic (58). A pocket exists close to this docking site on CDC25B to which potential inhibitors of this transient protein-protein interaction could be targeted by new drugs, and current efforts in many laboratories worldwide are directed toward this direction.
Although clarifying the details of transient protein-protein interactions involved in cell-cycle control is difficult and requires a combination of computational, genetic, biochemical and structural efforts, it could be a new frontier of discovery and development of inhibitors that can serve as cancer therapeutics.

Perspectives

In this review we tried to highlight the great potential that small molecule inhibitors of protein kinases and phosphatases have to treat human malignancies, by bringing some proof-of-concept examples that have proved successful in the last past few years. This is the reason why concerted efforts in the private sector and in the academia are currently directed, for instance, toward the identification and the high throughput screening of new inhibitors in tumor cell lines (59). In the coming years it is expected to see the appearance on the market of new validated and therapeutically efficient inhibitors of kinase, phosphatases, and of transient interactions of those with their substrates, which will improve the quality of life of patients with cancer.

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