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Therapeutic Targeting of PP2A

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Abstract

Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase that regulates many cellular processes. Given the central role of PP2A in regulating diverse biological functions and its dysregulation in many diseases, including cancer, PP2A directed therapeutics have become of great interest. The main approaches leveraged thus far can be categorized as follows: 1) inhibiting endogenous inhibitors of PP2A, 2) targeted disruption of post translational modifications on PP2A subunits, or 3) direct targeting of PP2A. Additional insight into the structural, molecular, and biological framework driving the efficacy of these therapeutic strategies will provide a foundation for the refinement and development of novel and clinically tractable PP2A targeted therapies.

Introduction

Phosphatases and kinases control the reversible phosphorylation of proteins, a central mechanism in the regulation of cellular signal transduction. The four classes of phosphatases are 1) protein serine/threonine phosphatases, 2) protein tyrosine phosphatases (PTPs), 3) dual specificity phosphatases, and 4) histidine phosphatases. The class of the phosphoprotein phosphatase (PPP) family is the largest of the serine/threonine family and includes PP1, PP2A, PP2B, PP4, PP5 and PP6 (Shi, 2009). The combined activity of the PPP family makes up the majority of the serine/threonine phosphatase activity within a cell.

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Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase that plays an important role in many cellular functions. PP2A is a heterotrimeric enzyme composed of a scaffolding subunit “A” (PP2A-A), regulatory subunit “B”, and catalytic subunit “C” (PP2A-C) (Figure 1). The A and C subunit form the core enzyme which interact with a B-subunit to create the holoenzyme. Both the A and C subunits have two different isoforms encoded by different genes, α and β (Figure 1A). The α and β isoforms of these subunits are highly homologous to one another, yet in the majority of cell types, the α isoform is predominantly expressed. The A subunit structure is composed of a series of 15 helical HEAT repeats, composed of antiparallel alpha helices (Groves et al., 1999). The unique stacking of these HEAT repeats, with a hinge region between HEATS 12 and 13, provides the scaffolding subunit with an inherent flexibility (Grinthal et al., 2010; Tsytlonok et al., 2013). These helices create a hydrophobic inner ridge, proposed to facilitate B and C subunit binding (Figure 1A&C). Supportive of this, residues along this hydrophobic ridge serve as important contact points between subunits in the solved crystal structures (Cho and Xu, 2007; Groves et al., 1999; Wlodarchak et al., 2013; Xu et al., 2006).

The C subunit shares a high degree of sequence and 3D structural homology with the catalytic subunits of its closely related family members (Cho and Xu, 2007; Shi, 2009) (Figure 1A). Similarly, the residues involved in catalysis, situated on the opposing face from the A subunit binding region, are among these highly conserved residues, contributing to the broad inhibitory function of molecules such as okadaic acid and microcystin. The catalytic activity of the C subunit is governed by the binding of two metal ions (presumably Mn^{2+}) and structural isomerization by the phosphotyrosyl phosphatase activator, PTPA (Cho and Xu, 2007; Guo et al., 2014). In addition to structural rearrangements and cofactor binding, the C-terminal tail of the C subunit is subject to a number of post-translational modifications which contribute to the B-subunit binding potential and enzymatic activity of the holoenzyme (Cho and Xu, 2007; Longin et al., 2007; Xing et al., 2008).

There are four classes of the B subunits, which include the B55 family (B), PR72 family (B'), B56 family (B''), and the Striatin family (B'''), each with multiple isoforms encoded for by different genes (Figure 1B). The structure of the regulatory subunits varies widely as exemplified in the solved crystal structures, allowing for broad and regulated substrate specificity involved in diverse cellular functions. For example, the structure of the B55 α subunit exhibits a propeller-like complex, while the B56 γ subunit mimics the A subunit's helical repeats, and the PR70 and PR72 subunits contain EF-hand calcium binding motifs that can adopt distinct orientations upon calcium binding (Cho and Xu, 2007; Magnusdottir et al., 2009; Wlodarchak et al., 2013; Xu et al., 2006). While the A and C subunits are ubiquitously expressed, the expression levels and cellular localization of the B subunits vary significantly across different cell types and tissues (Bononi et al., 2011; Flegg et al., 2010; Forester et al., 2007; Jin et al., 2009; Riedel et al., 2006; Slupe et al., 2011).

PP2A has been shown to regulate many vital cellular processes including, but not limited to, cell cycle, growth, metabolism, and apoptosis. PP2A dysregulation is commonly observed in many diseases including neurodegenerative disorders, cardiovascular pathologies, and cancer. In cancer, studies have highlighted the tumor suppressive capacity of PP2A. Concordant with these findings, PP2A is functionally inactivated in many cancers, typically

through one of several mechanisms including somatic mutation, loss of heterozygosity and/or decreased expression of PP2A subunits, increased expression of endogenous PP2A inhibitors and changes in phosphorylation/methylation of the C subunit (Cristóbal et al., 2015; Ion Cristóbal et al., 2014; Haesen et al., 2016; Janghorban et al., 2014; Liu et al., 2015; Nobumori et al., 2013; Rincón et al., 2015; Ruediger et al., 2011, 2001; Saddoughi et al., 2013; Shouse et al., 2016). Given its central role in regulating many key cellular processes and its role in human disease pathogenesis, many efforts have been developed to target PP2A.

Targeting of PP2A Inhibitors

Two endogenous inhibitors, I_1^{PP2A} and I_2^{PP2A} , were first identified as inhibitors of PP2A through isolation from bovine kidney (Li et al., 1995; Mei Li et al., 1996) (Table 1). I_2^{PP2A} would be later identified as SET, which was discovered to play a role in myeloid leukemogenesis and acute undifferentiated leukemia through the formation of the SET-CAN fusion gene (Adachi et al., 1994; von Lindern et al., 1992). Since its discovery, SET has been implicated in many cancers and its altered expression has been identified in acute myeloid leukemia (AML), chronic myeloid leukemia (CML), non-small cell lung cancer (NSCLC), and Wilms tumors (Liu et al., 2015; Makkinje, 1996; Neviani et al., 2005a) (Figure 2A). I_1^{PP2A} , or ANP32A, was identified to be a member of the SET complex. However, direct targeting of ANP32A and its role in cancer is less understood. The cancerous inhibitor of PP2A (CIP2A) is another endogenous inhibitor of PP2A that is overexpressed in human cancers (Figure 2A). CIP2A is a PP2A interacting protein that prevents PP2A mediated dephosphorylation of c-MYC at Ser 62 and AKT at Ser 473, resulting in the stabilization of c-MYC and increased AKT activity, respectively (Khanna et al., 2013; Sangodkar et al., 2015).

A growing body of literature has shown that antagonizing PP2A inhibitors has anti-tumor effects both *in vitro* and *in vivo* (Janghorban et al., 2014; Junttila et al., 2007; Neviani et al., 2005b). The discovery of small molecule antagonists of PP2A inhibitors, specifically CIP2A and SET, have provided insight into the mechanisms of PP2A dysregulation in cancer and highlighted a therapeutic avenue with promising potential (Table 2).

Targeting the PP2A Inhibitor SET

FTY720 and FTY720 Analogues

Ceramide and ceramide derivatives have been the best characterized method for targeting the PP2A/SET interaction for the treatment of cancer. Ceramide is a bioactive sphingolipid which can be synthesized by the cell and has been shown to activate PP2A through the disruption of the SET/PP2A interaction (Chalfant et al., 1999; Dobrowsky et al., 1993; Mukhopadhyay et al., 2009; Perry et al., 2012).

FTY720 (Fingolimod), a sphingosine analogue, is an FDA approved immunosuppressant used for the treatment of multiple sclerosis. *In vivo*, FTY720 is phosphorylated by sphingosine kinase-2 to produce FTY720-phosphate; the active immunosuppressant compound. The immunosuppressive activity of FTY720 results from its ability to bind the

sphingosine phosphate receptor (S1P1), leading to the internalization and degradation of this receptor (Brinkmann, 2009; Brinkmann et al., 2002; Mandala et al., 2002).

In addition to its immunosuppressive functions, FTY720 has been shown to be effective in multiple pre-clinical models of cancer including leukemia, colon cancer, non-small cell lung cancer, breast cancer, hepatocellular carcinoma, prostate cancer, and others (Chen et al., 2014; Liang et al., 2013; Matsuoka et al., 2003; Neviani et al., 2007; Saddoughi et al., 2013). Interestingly, the phosphorylation of FTY720 was not necessary for the anti-tumor effects, which have been attributed to its ability to disrupt the interaction of SET to PP2A, resulting in increased PP2A activity and cancer cell death (Neviani et al., 2007). The phosphorylation independent FTY720 activity has been further validated through the use of the chiral deoxy analogue, AAL(S), which is non-phosphorylatable. AAL(S) has been shown to retain the ability to activate PP2A in models of asthma and AML (Collison et al., 2013; Smith et al., 2016; Toop et al., 2016). This FTY720 dependent increase in PP2A activity resulted in the inactivation of multiple PP2A dependent substrates/pathways, including the pro-survival factors AKT and ERK which have subsequently been shown to mediate FTY720's antitumor effects (Matsuoka et al., 2003; Neviani et al., 2007; Shouse et al., 2016) (Figure 3A).

As stated above, PP2A is a complex protein with multiple holoenzymes and varying substrate specificity. To this end, researchers have tried to determine which holoenzymes are responsible for the anti-tumor effects of FTY720. In AML, it has been shown that B55 α expression increases with FTY720 treatment and reduced expression by both RNAi and loss of function mutations, rendered cells resistant to FTY720 treatment, indicating a potential role for the PP2A-B55 α holoenzyme in mediating FTY720 response (Ruvolo et al., 2014; Shouse et al., 2016; Yang et al., 2012).

Derivatives of FTY720, including MP07-66 and OSU-2S, have been found to exert equivalent antitumor effects, but did not alter the levels of circulating T lymphocytes or cause S1P1 internalization in mice, indicating that the immunosuppressive activity of these compounds was not required for the PP2A dependent antitumor activity of this series (Omar et al., 2011; Zonta et al., 2015) (Figure 3A). Additionally, it has been shown that OSU-2S has similar activity to FTY720 in both hepatocellular carcinoma and B-cell leukemia (Mani et al., 2015; Omar et al., 2011). These data suggest FTY720 analogues with reduced S1P/ immunosuppressive related side effects and enhanced anti-cancer efficacy could serve as important cancer therapeutics.

OP449 and ApoE Mimetics

Apolipoprotein E (ApoE) mimetics were first identified to have anti-inflammatory properties, and treatment with these compounds reduced NF- κ B pathway activation resulting in reduced phosphorylation of I- κ B (Singh et al., 2008). Further studies identified that the anti-inflammatory effects of the ApoE mimetics were not through the known ApoE receptor interaction (Christensen et al., 2011). These compounds, including COG112 and COG449 (OP449) were identified to bind the C terminal end of SET, disrupting the interaction between SET and PP2A, resulting in increased PP2A activity (Agarwal et al., 2014, 2011; Christensen et al., 2011) (Figure 3A). Of these derivatives, OP449 has been the most extensively studied in preclinical models of cancer and has been shown to have anti-

tumor effects in AML, breast cancer, and pancreatic cancer (Agarwal et al., 2014; Janghorban et al., 2014; Ramaswamy et al., 2015).

TGI1002

In a screening effort to find small molecules to overcome BCR-ABL mutation-mediated tyrosine kinase inhibitor (TKI) resistance, researchers discovered TGI1002 (Wang et al., 2015). This small molecule was found to disrupt the interaction between SET and PP2A and increase PP2A activity *in vitro*, and was shown to have anti-tumor effects in CML *in vivo* (Wang et al., 2015). The use of small molecules to disrupt the PP2A/SET interaction has promising therapeutic potential and further research into the mechanisms of how these molecules work may provide insight into the best preclinical models for their use.

Targeting the PP2A Inhibitor CIP2A

Bortezomib

Bortezomib (Velcade), an FDA approved proteasome inhibitor for the treatment of multiple myeloma and mantle cell lymphomas, has been suggested to have proteasome-independent activity mediated through PP2A (Niesvizky et al., 2015). Traditionally, the main functions of Bortezomib have been attributed to its ability to inhibit the 26S subunit of the proteasome, leading to cell cycle arrest and apoptosis (Adams and Kauffman, 2004). Recent observations suggest that Bortezomib may have additional mechanisms of action. First, cancer cells which exhibited equal proteasomal inhibition displayed a differential apoptotic response to Bortezomib (Chen et al., 2008; Liu et al., 2013; Tseng et al., 2012). Second, Bortezomib derivatives with diminished proteasomal inhibition showed equal or increased cytotoxicity to cancer cells (Hou et al., 2013). Additional evidence suggested that this secondary mechanism of action was through a loss of CIP2A expression in multiple cancer types (hepatocellular carcinoma, head and neck squamous cell carcinoma, triple negative breast cancer, and a variety of leukemias), resulting in increased PP2A activity (Chen et al., 2010; Ding et al., 2014; Hou et al., 2013; Lin et al., 2012; Liu et al., 2013) (Figure 3A). Furthermore, Bortezomib's cytotoxic function was blunted by siRNA mediated loss of PP2A-C subunit, supporting the role of PP2A in mediating Bortezomib-induced cytotoxicity (Lin et al., 2012). The loss of CIP2A upon Bortezomib treatment occurs at a transcriptional level, however the mechanism of transcriptional regulation of CIP2A by Bortezomib remains unclear (Chen et al., 2010; Huang et al., 2012; Lin et al., 2012; Liu et al., 2013).

Molecular studies of PP2A substrates have identified AKT dephosphorylation at Ser 473 to be required for Bortezomib's apoptotic effects (Chen et al., 2010, 2008; Lin et al., 2012). Additionally, the anti-cancer effect of Bortezomib is blunted through the induction of AKT activity via EGFR activation or through the expression of constitutively active AKT. Preclinical studies have characterized Bortezomib as an effective treatment to sensitize solid tumors towards radiation therapy through its ability to negatively regulate AKT signaling (Chen et al., 2011; Huang et al., 2012).

Erlotinib

Erlotinib (Tarceva) is an FDA approved EGFR kinase inhibitor indicated for the treatment of EGFR mutant non-small cell lung cancers (Mendelsohn and Baselga, 2006; Wu et al., 2015). Interestingly, several observations indicate Erlotinib may have an EGFR-independent mechanism of action. First, in a phase II all comers clinical trial, a small subset of EGFR wild-type patients also respond to Erlotinib treatment (Cappuzzo et al., 2010; Ciuleanu et al., 2012; Wang et al., 2014). Second, Erlotinib derivatives, which were derived to remove their EGFR activity and retain their effects on CIP2A, exhibited increased cytotoxic effects when compared to Erlotinib (Chao et al., 2014; Chen et al., 2012; Yu et al., 2014). Several of these derivatives have shown the ability to transcriptionally downregulate CIP2A expression (Chao et al., 2014; Chen et al., 2012; Liu et al., 2017; Wang et al., 2014; Yu et al., 2014) (Figure 3A). In these studies, Erlotinib derivatives induced decreased nuclear expression of a single transcription factor, Elk-1, resulting in less Elk-1 binding at the CIP2A promoter. However, the mechanism by which these compounds impact Elk-1 nuclear expression has yet to be elucidated. Some evidence suggests that the observed effects on CIP2A could result from downstream ERK inhibition leading to Elk-1 inactivation (Liu et al., 2017). Elucidating the precise molecular mechanism of action of this drug series will improve our understanding of mechanisms regulating CIP2A expression and function, PP2A activity, and assist in the development of CIP2A targeting agents.

Celastrol

Celastrol is a compound found in traditional Chinese medicine named *Tripterygium wilfordii* (Liu et al., 2011). Celastrol has been shown to have anticancer properties in multiple cell types which have been largely attributed to Celastrol's ability to engage mitochondrial-induced apoptotic signaling, however, the effector target of these mitochondrial signaling cascades has yet to be elucidated (Lee et al., 2012; Mou et al., 2011; Shrivastava et al., 2015; Yang et al., 2011; Yu et al., 2015). Multiple groups have identified changes in CIP2A expression levels upon Celastrol treatment, which correlate with changes in downstream CIP2A targets, such as phosphorylated AKT and c-MYC levels (Liu et al., 2014; Shrivastava et al., 2015; Wu et al., 2017). Unlike the previously mentioned CIP2A modulating agents, Celastrol has been shown to regulate CIP2A directly, by binding and promoting its interaction with CHIP, the ubiquitin E3 ligase responsible for mediating CIP2A's proteasomal degradation (Liu et al., 2014). In its entirety, the data is suggestive of CIP2A as a direct target of Celastrol, however more work is required to link Celastrol's regulation of CIP2A with its apoptotic activity (Figure 3A). Additionally, Celastrol has been shown to induce cell death through multiple apoptotic pathways, including both caspase-dependent and caspase-independent pathways. Identifying whether CIP2A mediates both of these signaling cascades or if additional targets may play a role in the observed anti-cancer activities will provide insight into CIP2A biology and the use of Celastrol as a therapeutic agent (Yang et al., 2011).

Targeting of PP2A Post Translational Modifications

The functional implications of post-translational modifications (PTMs) on PP2A, have yet to be fully characterized and remain of great interest in the field. Global mass spectrometry

analysis and/or biochemical studies have highlighted three particular PTM's on the C terminal region of the C-subunit which regulate its composition, substrate specificity, and/or enzymatic function; 1) phosphorylation at the Threonine 304 (T304) residue, 2) phosphorylation at the Tyrosine 307 (Y307) residue and, 3) the unique methylation of a carboxy-terminal Leucine 309 (L309) residue (Evans et al., 1999; Groves et al., 1999; Guo et al., 2014; Jennifer B. Jackson and Pallas, 2012; Longin et al., 2007; Ortega-Gutierrez et al., 2008; Xing et al., 2008; Yu et al., 2001).

The regulatory kinases and subsequent functional impact of T304 phosphorylation have yet to be fully characterized, although during mitosis, CDK1 has been implicated as a kinase capable of phosphorylating this residue (Schmitz et al., 2010). Additionally, mutational perturbations to the T304 residue demonstrate that phosphorylation at this site regulates B subunit binding. For example, T304 phosphomimetics demonstrated decreased B55 binding without affecting L309 methylation (Longin et al., 2007).

Possibly the most widely published marker of PP2A inactivation and regulatory subunit regulation is the proposed Y307 phosphorylation site. Identified in biochemical assays, phosphorylation at Y307 has been implicated as a marker of PP2A inactivation and thought to inversely correlate with disease prognosis in select hematologic malignancies and solid tumors (I Cristóbal et al., 2014; Farrell et al., 2015; Neviani et al., 2005a; Perrotti and Neviani, 2013; Rincón et al., 2015). Work in biological systems including cellular assays, mouse work, and patient samples, have mainly utilized immunoblotting to identify phosphorylation at this residue with one of three western blot approved antibodies (Abcam, Santa Cruz, R&D) or one antibody validated for ELISA (Millipore). Unfortunately, the Abcam antibody, clone E155, has recently been found by the distributor to recognize total PP2A-C independently of Y307 phosphorylation status, a notable finding that must be considered when interpreting previously published reports that utilized this antibody.

Finally, methylation of the L309 residue of the catalytic subunit has been shown to influence the formation of B-subunit containing holoenzymes. For example, B55 isoforms appear to require L309 methylation in order to bind the core dimer, B56 regulatory subunits are able to bind to either form of L309 with some isoforms preferring methylated L309, and the PR72 and Striatin classes of regulatory subunits show no preference for either form of L309 (Jennifer B. Jackson and Pallas, 2012; Longin et al., 2007; Yu et al., 2001). These biological observations are further explained by the structural insights deemed from the crystal structure of PP2A heterotrimers. Crystal structure data of a B56 γ containing heterotrimer demonstrated how the negatively charged carboxyl group of the non-methylated L309 prevented the C-terminal tail from interacting with the E64 residue of the scaffolding A subunit (Cho and Xu, 2007). These observations highlight the importance of L309 methylation in regulating holoenzyme composition and thus regulation of the tumor suppressive functions attributed to PP2A. This critical regulatory mechanism has thus become an area of interest within PP2A-directed therapeutics as the sole esterase responsible for removing this carboxyl-methylation, Protein Methyl-Esterase 1 (PME-1) (Figure 2B and Table 1), is dysregulated in a range of cancers.

PME-1 overexpression occurs in a broad range of malignancies and increased PME-1 activity is associated with increased cellular growth and *in vivo* tumorigenicity (Li et al., 2014; Pusey et al., 2014; Puustinen et al., 2009; Wandzioch et al., 2014 Kaur et al.). Additionally, PME-1 expression directly correlates with disease progression in human astrocytic gliomas, while driving kinase inhibitor resistance in glioma cells (Kaur et al., 2016; Puustinen et al., 2009). Functional studies suggest PME-1 overexpression results in the enhanced activation of numerous oncogenic signaling cascades including ERK, AKT, and WNT (Jackson and Pallas, 2012; Puustinen et al., 2009; Wandzioch et al., 2014). These observations serve as the basis for the potential role of PME-1 as a biomarker and therapeutic target in cancer (Table 2).

The mechanisms by which PME-1 regulates PP2A continues to be an area of active research. The most well-studied role of PME-1 is its esterase activity, functioning to demethylate the L309 residue of PP2A-C (Figure 2B). Recent insights into the structural interaction between PME-1 and the PP2A catalytic subunit suggest additional mechanisms by which PME-1 directs PP2A activity. It has been shown that in addition to demethylating L309, PME-1 binds directly to the catalytic active site of PP2A-C and is able to eject the requisite Manganese ions from the PP2AC catalytic site (Xing et al., 2008).

Countering PME-1 activity is the Leucine Carboxyl Methyl-Transferase 1 (LCMT1), which utilizes S-adenosylmethionine (SAM) as a substrate to transfer a methyl group onto the terminal carboxyl group of the L309 residue (Figure 2B and Table 1). The specificity of this unique type of terminal carboxyl methylation restricts LCMT-1 and PME-1 activity to the phosphoprotein phosphatase enzymes PP2A, PP4, and PP6, further highlighting their potential as selective therapeutic targets. Interestingly, LCMT-1 knockdown is able to facilitate cellular transformation *in vitro*, further implicating its importance in regulating the tumor suppressive functions of PP2A (Jennifer B Jackson and Pallas, 2012). LCMT-1 dysregulation in human cancer is currently under-explored and represents an important gap in the mechanistic understanding of PP2A dysregulation in cancer.

Pharmacological Inhibition of PP2A De-methylation

Utilizing a novel PME-1 activity assay designed for high throughput screening, Bachovchin et al. identified a class of aza- β -lactams (ABLs) as potent, covalent inhibitors of PME-1 catalytic activity (Figure 3B). Despite demonstrating preserved methylation of PP2A, both in cell-free and cellular systems, as well as the induction of apoptosis in cells treated with ABLs, the pre-clinical efficacy of this molecule series in tumor models has yet to be fully explored (Bachovchin et al., 2011). Additional exploration of PME-1 targeted therapeutics for the management of neurodegenerative diseases led to the identification of another distinct class of PME-1 inhibitors. Investigating the negative correlation between coffee consumption and neurodegenerative diseases, Lee and colleagues demonstrated that the neuro-protective compound, eicosanoyl-5-hydroxytryptamide (EHT), inhibits PME-1 and facilitates the methylation of PP2A in cellular systems (Lee et al., 2011). Despite the preclinical data supporting a role for EHT in disrupting PME-1's regulatory role on PP2A, its clinical potential has also not been fully explored.

Recent evidence suggests both methyl esterase-dependent and methyl esterase-independent mechanisms for PME-1 dependent inhibition of PP2A (Ogris et al., 1999; Puustinen et al., 2009; Wandzioch et al., 2014; Yabe et al., 2015). With this in mind, the development of clinically translatable PME-1 directed therapies may need to target both posited mechanisms of PME-1 dependent regulation of PP2A.

Promoting Methylation

To date, there have been no published attempts to directly target the highly specific methyltransferase, LCMT1. Although there are select agents, such as the DNA damaging agent chloroethylnitrosourea (CENU), which appeared to induce apoptosis and PP2A methylation, which is reversible with okadaic acid treatment, (Guénin et al., 2008), the exact mechanism for the reported activity of these compounds and their dependence on PME-1 versus LCMT-1 has yet to be established. Future studies aimed at understanding the control mechanisms governing PP2A methylation will be crucial to help guide the development of future therapies targeting PP2A-methylating factors.

Direct Targeting of PP2A

SMAPs and Phenothiazines

While the majority of therapeutics target PP2A indirectly, recent studies have uncovered small molecules, such as phenothiazines, that directly activate PP2A (Figure 3C and Table 2). Phenothiazines have traditionally been used as potent dopamine receptor antagonists for the treatment of various psychiatric disorders (Delay, J., Deniker, P. & Harl, 1952; Feinberg and Snyder, 1975; Sammut et al., 2007; Snyder et al., 1974; Sudeshna and Parimal, 2010). While several studies have reported that phenothiazines also display anti-tumor effects (Choi et al., 2008; Gu et al., 1999; López-Girona et al., 1992; Motohashi, 1991; Sangodkar et al., 2012), Gutierrez et al elucidated a PP2A dependent mechanism by which these FDA approved drugs induced apoptosis (Gutierrez et al., 2014). In a small molecule screen aimed at identifying compounds with activity toward c-MYC driven T-ALL, perphenazine induced apoptosis in fish, mouse, and human T-ALL cells. Characterization of perphenazine using ligand affinity chromatography and mass spectrometry identified PP2A as the molecular target of this drug series. Treatment of T-ALL cells with perphenazine led to dephosphorylation of many PP2A targets including AKT. Furthermore, targeted knockdown of the PP2A A or C subunits attenuated perphenazine's activity validating PP2A's role in mediating cell death. These studies showed the promising potential of directly activating PP2A through a small molecule based approach. However, their pronounced extrapyramidal and anti-cholinergic effects limited the further pursuit of phenothiazines and related dibenzazepines for cancer treatment. To explore the full potential of antipsychotics as anticancer agents, the tricyclic neuroleptics were reengineered to decouple the CNS pharmacology from the anti-proliferative properties of this drug class (Kastrinsky et al., 2015; Sangodkar et al., 2017). Iterative rounds of synthesis and optimization led to the development of a class of small molecule activators of PP2A (SMAPs) (Kastrinsky et al., 2015).

SMAP treatment of lung cancer cell lines induced apoptosis and decreased the phosphorylation of PP2A targets such as ERK (Sangodkar et al., 2017). PP2A dependent inhibition of tumor growth using xenograft and transgenic mouse models of tumorigenesis further demonstrated the therapeutic potential of SMAP therapy. Molecular analysis of these tumors showed induction of apoptosis and dephosphorylation of targets of PP2A. Biochemical studies showed that SMAPs bound to the PP2A-A subunit and induced a conformational change in PP2A. Mutation of the putative drug binding sites on the scaffolding subunit or PP2A inactivation by SV40 small T expression resulted in cellular and in vivo resistance to SMAPs, further confirming PP2A as the direct target of this series of small molecules. Future studies elucidating the tumor suppressive sequence of events induced by SMAP-activated PP2A will be crucial in determining the most effective application of this approach.

LB-100

LB-100 is a small molecule inhibitor of the PP2A-C subunit and has recently completed a Phase I clinical trial for the treatment of solid tumors in combination with Docetaxel (Chung et al., 2016) (Figure 3C and Table 2). Despite strong evidence characterizing PP2A as an important tumor suppressor, preclinical data identified LB-100 as an effective radio- and chemo- sensitizer. LB-100 has been validated as a radio-sensitizer in preclinical models of pancreatic cancer, glioblastoma, and nasopharyngeal carcinoma (Gordon et al., 2015; Lv et al., 2014; Wei et al., 2013). This sensitization has been attributed to inhibition of homologous recombination repair (HRR) (Wei et al., 2013). The HRR inhibition upon LB-100 treatment has been shown to be partially through increased phosphorylation of the PP2A substrate CDC25C at the threonine 130 site, resulting in activation of CDK1, G2/M accumulation, and ultimately mitotic catastrophe and apoptosis (Gordon et al., 2015; Lv et al., 2014).

LB-100's role as a chemo-sensitizer has been established in multiple preclinical models of brain cancer, ovarian cancer, sarcoma, pancreatic cancer, and hepatocellular carcinoma, tested in combination with both cisplatin and doxorubicin (X.-L. Bai et al., 2014; X. Bai et al., 2014; Chang et al., 2015; Ho et al., 2016; Lu et al., 2009; Zhang et al., 2015). Multiple studies have reported treatment of LB-100 lowers the cell cycle arrest induced by chemotherapy (Chang et al., 2015; Ho et al., 2016; Zhang et al., 2015). Additionally, LB-100 was shown to lower MAD2 spindle checkpoint expression and CHK-1/2 phosphorylation, which inhibited DNA damage response signaling and resulted in mitotic catastrophe after treatment with cisplatin (Zhang et al., 2015).

Additionally, combination treatment of LB-100 and doxorubicin caused an increase in xenograft tumor blood flow and increased accumulation of doxorubicin in the tumors, which correlated with an increase in VEGF secretion and HIF-1 α expression (X.-L. Bai et al., 2014; X. Bai et al., 2014). Subsequently, the effects of LB-100 on vascular permeability could result in unwanted side effects including increased metastasis, from the compromised vascular membrane, or increased tumor growth, from an increased nutrient supply, which is supported by single-agent treated xenografts resulting in a subtle but significant growth increase (X.-L. Bai et al., 2014).

In aggregate, these studies support the use of LB-100 as an effective sensitizer for both radiation and chemotherapies through the dysregulation of PP2A-mediated cell cycle checkpoints. In preclinical models, the combination of DNA damaging agents and PP2A inhibition allowed for increased mitotic stress and increased cell death.

Other Mechanisms of Targeting PP2A

There are a select group of compounds that undoubtedly induce cell death in a PP2A dependent manner, but the exact mechanisms of PP2A activation remains unclear. This list includes xylulose 5-phosphate, carnosic acid, α -tocopheryl succinate and forskolin (Figure 3C and Table 2).

Xylulose 5-phosphate (X5P), a nucleotide precursor produced by the pentose phosphate pathway, added to fractionated hepatic murine lysates demonstrated an increase in the free phosphate resulting from X5P-induced PP2A activity (Kabashima et al., 2003). These observations are consistent with other nutrient dependent experiments that showed glutamine was able to preferentially activate specific PP2A holoenzyme complexes (Reid et al., 2013). Additional insight into the mechanisms of PP2A regulation by nutrient density and nucleotide precursors such as X5P could inform more specific applications for PP2A directed therapies in cancer.

Carnosic acid (CA), a polyphenolic diterpene, isolated from the rosemary plant (*Rosemarinus officinalis*), has been shown to inactivate the AKT/IKK/NF- κ B pathway in a PP2A dependent manner resulting in the induction of apoptosis in androgen-independent prostate cancer cells (Kar et al., 2012). Conversely, treatment of skeletal muscle cells with CA results in the demethylation of PP2A and activation of the AKT pathway to enhance glucose uptake (Lipina and Hundal, 2014). This discordance in therapeutic response, even within a single pathway modulated by PP2A, highlights the importance of elucidating the mechanism of actions of these PP2A-modulating strategies in order to leverage these findings towards a better understanding of PP2A biology and its potential for drug development approaches.

α -Tocopheryl succinate (α -TOS), a Vitamin E analog, has also been shown to induce cell death in cellular models of both hematopoietic and solid tumors. Neuzil and colleagues demonstrated α -TOS induced apoptosis in colon cancer cells resulted from multiple cellular effects including lysosomal and mitochondrial de-stabilization through the PP2A-dependent dephosphorylation of PKC α and inactivation of BCL2 (Neuzil et al., 2002). Additionally, Huang et al, investigated the therapeutic potential of α -TOS in an androgen dependent prostate cancer model and found that it induced PP2A dependent inactivation of JNK and subsequent proteasome mediated degradation of the transcription factor Sp1, resulting in cell death (Huang et al., 2009).

Forskolin is a naturally occurring diterpenoid, derived from *Coleus forskohlii* root. Classically, forskolin is recognized as an adenylate cyclase activator which drives elevated levels of intracellular cAMP (Seamon et al., 1981). Forskolin therapy has been shown to have both PKA/adenylate cyclase dependent and independent mechanisms of inducing cell death (Neviani et al., 2005a; Seamon et al., 1981). With regards to PP2A, forskolin treatment

results in the dephosphorylation of PP2A substrates such as EF-2 and RB, which are reversed by the addition of okadaic acid, but not rescued by the PP1 selective inhibitor tautomycin (Feschenko et al., 2002). Additionally, the cAMP inactive forskolin analogue, 1,9 dideoxyforskolin, induces the same pattern of PP2A substrate dephosphorylation and okadaic acid rescue, further suggesting that the cellular effects are independent of the canonical cAMP signaling attributed to forskolin (Feschenko et al., 2002; Neviani et al., 2005a; Seamon et al., 1981). Whether the forskolin-induced dephosphorylation of PP2A substrates is due to direct or indirect activation of PP2A holoenzymes or regulatory proteins has yet to be determined.

Conclusion

Protein phosphatase 2A (PP2A) is a family of serine/threonine phosphatases subject to a number of regulatory mechanisms that govern the cellular localization, substrate specificity, and the enzymatic potential of this enzyme family. Although its complex regulation endows PP2A with broad cellular responsibilities, it also makes this tumor suppressor vulnerable to many different mechanisms of pathologic dysregulation. Elucidating the mechanisms of PP2A dysregulation in disease has led to growing interest in the development of PP2A targeted therapeutics. Early efforts have highlighted the potential of PP2A targeted therapeutic strategies in cancer, but the complexity of PP2A regulation emphasizes the need for further refinement and the potential of multi-faceted approaches. Despite the promising potential demonstrated by the strategies outlined above, these investigations illustrate new questions pertaining to PP2A biology and regulation. Continued investigation of the mechanisms of PP2A regulation, holoenzyme formation, and subsequent signaling pathway modulation will be fundamental in determining effective PP2A modulating therapeutic strategies to target PP2A in specific disease contexts.

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Figure 1. Structure of protein phosphatase 2A (PP2A)

The Protein Phosphatase 2A (PP2A) holoenzyme is composed of three subunits. A) The scaffolding subunit A (left) exists in two isoforms, Aα and Aβ, and are encoded by separate genes. The A subunit binds both the B and C subunits through its flexible 15 consecutive HEAT-repeat helical structure (PDB code: 2IAE). The catalytic subunit C (right) also exists in two isoforms, Cα and Cβ, and are encoded by separate genes. Both of the isoforms of the C subunit contain conserved C - terminal domain that undergoes post-translational modification as a regulatory mechanism (PDB code: 2IAE). B) The regulatory subunits

consist of 4 unique classes of proteins: B (PDB code: 3DW8), B' (PDB code: 2IAE), B'' (PDB code: 4I5L), and B''', which have not been crystallized. Within each class, multiple isoforms exist and each isoform is encoded by a separate gene. C) The core enzyme structure (left) consists of A α subunit (blue) and C α subunit (pink) (PDB code: 2IE3). One of the solved PP2A holoenzyme structures (right) with A α (in blue) subunit, B' subunit (in yellow), and C α (in pink) subunit (PDB code: 2IAE).

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Figure 2. Endogenous Regulation of PP2A

A) Endogenous inhibitors of PP2A (Red) have evolved to target the PP2A holoenzyme (PDB code: 2IAE). Endogenous activators, such as ceramide (green), counteract some of the endogenous inhibitors. B) LCMT1 (in green) utilizes S-adenosylmethionine (SAM) as a substrate to transfer a methyl group onto the terminal carboxyl group of the L309 residue of C subunit (in pink). PDB code: 3P71. PME-1 (in red) regulates PP2A activity by demethylating the PP2A-C subunit (PP2A A subunit shown in blue). PDB code: 3C5W.

Figure 3. Exogenous PP2A modulators

A) Several groups have developed ways to target PP2A indirectly by binding and inhibiting SET and CIP2A (blue). An additional group of drugs function through the transcriptional down-regulation of CIP2A (left). B) Compounds, such as ABL-127, have been developed to inhibit PME-1 methyltransferase activity. Inhibition of PME-1 using this strategy results in an increase or stabilization of L309 methylation levels on the PP2AC subunit. C) Two classes of molecules have been shown to directly bind to PP2A. The first class, SMAPs and perphenazine, bind the A subunit and activate the phosphatase. The second class, LB-100

binds to the C subunit and inhibits PP2A catalytic activity. Additional molecules are thought to modulate PP2A activity through unknown or indirect mechanisms.

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Table 1

Endogenous Regulators of PP2A

Protein	Function	Mechanism	References
<i>CIP2A</i>	Inhibitor	Binds to PP2A, preventing the dephosphorylation of c-MYC and AKT	(Junttila et al., 2007)
<i>SET</i>	Inhibitor	Binds to and inactivates PP2A	(Li et al., 1996; Makkinje, 1996)
<i>PME-1</i>	Regulator/Inhibitor	Demethylation of L309 on the PP2A C subunit	(Ogris et al., 1999; Ortega-Gutierrez et al., 2008; Puustinen et al., 2009; Sontag et al., 2004; Wandzioch et al., 2014; Xing et al., 2008; Yabe et al., 2015)
<i>LCMT-1</i>	Regulator	Methylation of L309 on the PP2A C subunit	(De Baere et al., 1999; Guo et al., 2014; Jennifer B. Jackson and Pallas, 2012; MacKay et al., 2013; Nicholls et al., 2016; Sontag et al., 2004)
<i>PTPA</i>	Activator	Binds to PP2A dimer, and may help reactivate PME-1 bound PP2A C	(Fellner et al., 2003; Guo et al., 2014; Longin et al., 2004)
<i>Ceramide</i>	Activator	Prevents binding of SET to PP2A	(Dobrowskyss et al., 1993; Mukhopadhyay et al., 2009; Perry et al., 2012)

Table 2

Strategies for Targeting PP2A

Target	Mechanism	Drugs/Compounds	References
<i>CIP2A</i>	Decrease CIP2A expression (through decreased transcription or increased degradation)	Erlotinib, Celestrol, Bortezomib, TD-19, TD-44	(Chao et al., 2014; Chen et al., 2012, 2010; Ding et al., 2014; Hou et al., 2013; Liu et al., 2017, 2013, 2014; Tseng et al., 2012; Wang et al., 2014; Wu et al., 2017; Yu et al., 2014, 2013)
<i>SET (I2PP2A)</i>	Prevents binding of SET to PP2A	COG112, OP449,, FTY720, OSU-2S, MP07-66, TGI1002	(Matsuoka et al., 2003; Neviani et al., 2007; Omar et al., 2011; Zonta et al., 2015)
<i>PME-1</i>	Bind and inhibit catalytic activity of PME-1	ABL-127	(Bachovchin et al., 2011)
<i>PP2A A Subunit</i>	Direct binding to PP2A A subunit resulting in conformational change and PP2A activation	SMAPs, Phenothiazines	(Gutierrez et al., 2014; Kastrinsky et al., 2015; Sangodkar et al., 2017)
<i>PP2A C Subunit</i>	Binds and inhibits catalytic activity of PP2A	LB-100	(Chang et al., 2015; Gordon et al., 2015; Ho et al., 2016; Hong et al., 2015; Lv et al., 2014; Wei et al., 2013; Zhang et al., 2015)
<i>Other Targets</i>	Unknown	Forskolin, Vitamin E analogs, Lanolinamide, Canthardin	(Feschenko et al., 2002; Huang et al., 2009; Kar et al., 2012; Neuzil et al., 2002; Neviani et al., 2005a; Seamon et al., 1981)