

## Molecular Pathways: PI3K Pathway Phosphatases as Biomarkers for Cancer Prognosis and Therapy

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### Abstract

Cancer research has seen tremendous changes over the past decade. Fast progress in sequencing technology has afforded us with landmark genetic alterations, which had immediate impact on clinical science and practice by pointing to new kinase targets, such as phosphoinositide 3-kinase (PI3K), the EGF receptor, or BRAF. The PI3K pathway for growth control has emerged as a prime example for both oncogene activation and tumor suppressor loss in cancer. Here, we discuss how therapy using PI3K pathway inhibitors could benefit from information on specific phosphatases, which naturally antagonize the kinase targets. This PI3K pathway is found mutated in most cancer types, including prostate, breast, colon, and brain tumors. The tumor-suppressing phosphatases operate at two levels. Lipid-level phosphatases, such as PTEN and INPP4B, revert PI3K activity to keep the lipid second messengers inactive. At the protein level, PHLPP1/2 protein phosphatases inactivate AKT kinase, thus antagonizing mTOR complex 2 activity. However, in contrast with their kinase counterparts the phosphatases are unlikely drug targets. They would need to be stimulated by therapy and are commonly deleted and mutated in cancer. Yet, because they occupy critical nodes in preventing cancer initiation and progression, the information on their status has tremendous potential in outcome prediction, and in matching the available kinase inhibitor repertoire with the right patients. *Clin Cancer Res*; 20(12); 3057–63. ©2014 AACR.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### CME Staff Planners' Disclosures

The members of the planning committee have no real or apparent conflict of interest to disclose.

### Learning Objectives

Upon completion of this activity, the participant should have a better understanding of the lipid and protein phosphatases that regulate the PI3K pathway and their potential application as biomarkers to improve patient stratification, prediction of therapeutic responses, and disease outcomes in patients with cancer.

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### Background

#### The PTEN/PI3K pathway

PTEN was discovered in 1997 as the result of a chase for the candidate tumor suppressor in the frequently deleted chromosome 10q23 region (1, 2). The two teams immedi-

ately saw that the gene encodes a phosphatase, which launched a flurry of investigations for its substrate. In spite of the logical appeal for a phosphatase tumor suppressor to reverse the action of an oncogenic protein kinase, a landmark study identified the PTEN substrate to be the membrane phospholipid phosphatidylinositol 3,4,5 trisphosphate [PI(3,4,5)P<sub>3</sub>; ref. 3]. Because PTEN showed specificity for removing the phosphate at the 3-position of the inositol ring [creating PI(4,5)P<sub>2</sub>], it immediately became clear that its activity antagonizes the previously identified class I PI3Ks, which conversely phosphorylate the PI(4,5)P<sub>2</sub> lipid at that position (ref. 4; reviewed in ref. 5). These results gave birth to our current concept of PTEN and the class I PI3Ks as top-level communicators of growth control in

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cancer (see Fig. 1). Today we know that this pathway constitutes the major oncogenic signaling axis next to the RAS/mitogen-activated protein kinase pathway. In this review, we discuss how the lipid and protein-level phosphatases cooperate to protect from cancer and how their use as biomarkers could assist outcome prediction and therapy approach.

PI3Ks constitute a conserved family of lipid kinases that phosphorylate phosphoinositides (PI) at the 3-position of their inositol head group (5). The family is classified into several subtypes depending on the substrate PIs that they can phosphorylate, yet the class I PI3Ks are unique: Only they can create the master growth control second messenger, PI(3,4,5)P<sub>3</sub> (below termed PIP<sub>3</sub>). The class IA PI3Ks relay extracellular growth and survival signals into the cell by producing PIP<sub>3</sub> after activation by ligand bound receptor tyrosine kinases. The PIP<sub>3</sub> lipid then attracts proteins such as AKT kinase and its activating kinase 3-phosphoinositide-dependent protein kinase 1 via their Pleckstrin homology domains, thus converting the lipid-phosphorylation code into protein signaling cascades (see Fig. 1). Accordingly, tumor suppression by the pathway's phosphatases occurs at two fundamentally different levels: Lipid-level phosphatases convert the actively signaling PIP<sub>3</sub> lipids to their inactive isoforms, and protein-level phosphatases inactivate the downstream phosphorylated proteins back to the nonphosphorylated state. Below, we discuss the functions and interactions among the pathway's major phosphatases, and their potential in predicting disease outcomes and therapy response.

### The lipid-level phosphatases

The phosphatidylinositol membrane lipids (PIP) constitute only a very small percentage of total membrane lipid mass, consistent with the notion that they do not define physical membrane properties but instead serve as top-level intracellular second messengers for signaling (6). Of the seven naturally occurring PIP<sub>n</sub> phospho-isoforms, PIP<sub>3</sub> executes the major known signaling function in cancer. However, PIP<sub>3</sub> is only present at very low levels in cell membranes (6), reflecting the transient nature of signaling at the level of the lipids. Keeping these second messenger levels low is the major known function of the phosphatase PTEN (7).

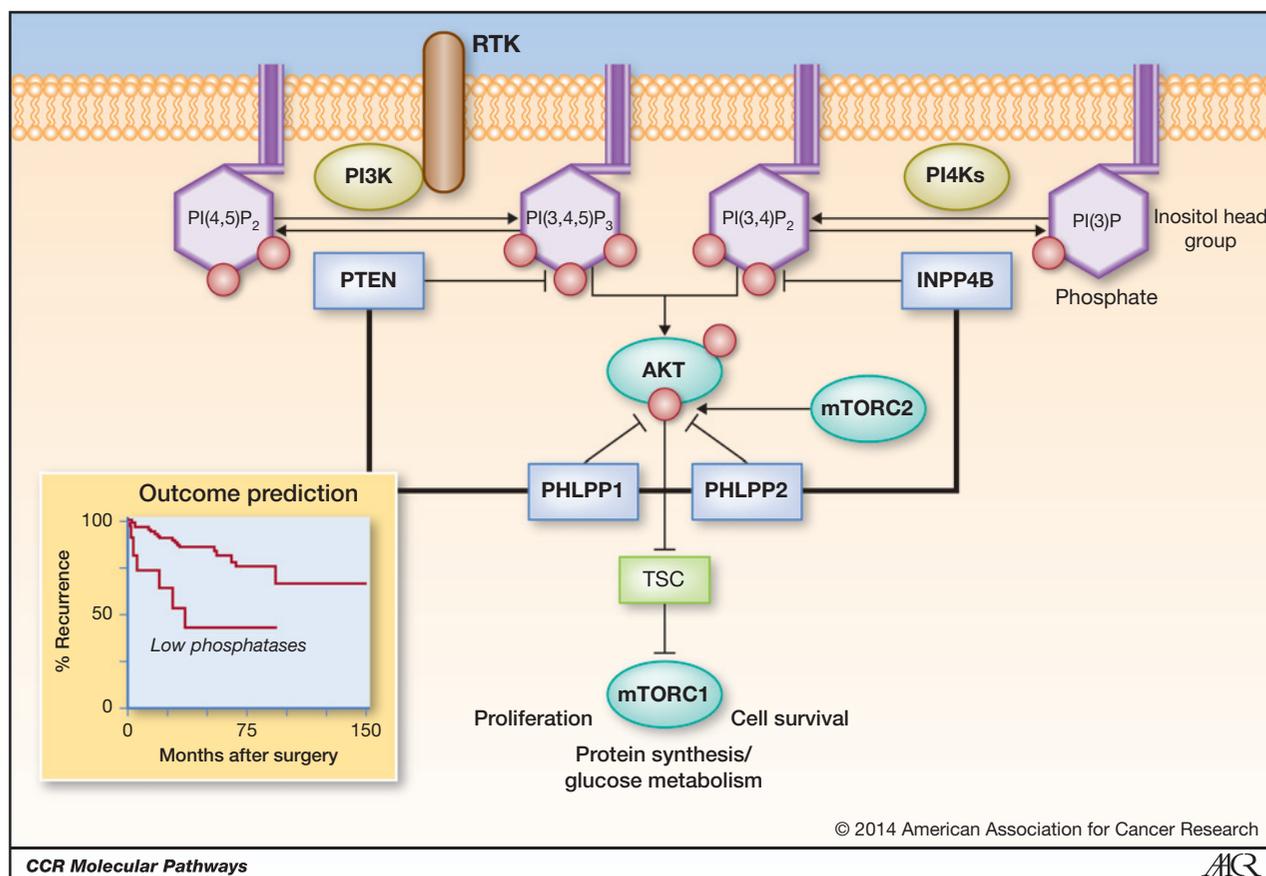
**PTEN.** PTEN occupies a unique position in antagonizing PI3Ks (see Fig. 1) by dephosphorylating PI(3,4,5)P<sub>3</sub> to PI(4,5)P<sub>2</sub>. To date, it represents the most efficient suppressor of the PI3K pathway. *In vitro*, it inhibits cell growth, survival, and proliferation so efficiently that stable expression in cancer cells is very hard to achieve. The *PTEN* gene has been mutated in heritable cancer syndromes, which are now collectively referred to as the PTEN hamartoma tumor syndromes, with Cowden disease, Proteus-like and Bannayan-Riley-Ruvalcaba syndromes showing the highest PTEN germline mutation frequency (80%, 60%, and 60%, respectively; ref. 8). Somatic *PTEN* alterations are seen in such a high number of human epithelial cancers that they rival those of p53 (9), see also the COSMIC database, <http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln = PTEN>). However, deregulation of PTEN that does not involve gene alterations is also of

critical importance in cancer (10, 11). Primary human prostate cancers, for example, frequently present with partial reduction of the PTEN protein but rarely with complete gene inactivation (12–14). Animal models have shown that *Pten* is haploinsufficient in prostate and other cancer types (15–18). Given the prominent role of PTEN in cancer signaling, this led to the realization that the classical "two-hit-hypothesis" for tumor suppression (19) needed to be expanded (reviewed in ref. 20). Analysis of PTEN alteration in surgically removed prostate cancers has revealed that 86% of tumors retain the gene whereas protein loss or strong reduction is found at 75% frequency (13). Similar observations were made in colorectal and lung cancer (21, 22), where PTEN protein loss is far more frequent than gene/RNA loss, and in thyroid cancer, endocrine pancreatic tumors, and melanoma, where PTEN is often lost from cell nuclei (ref. 23–25; reviewed in ref. 26). These cases mostly exhibit normal RNA levels, suggesting that PTEN protein degradation is a common cause of cancer formation (reviewed in ref. 26). The finding that homozygous *PTEN* loss triggers p53-mediated senescence explained why the partial loss is widespread in cancer cells with functional p53 (27). In prostate cancer, for example, p53-deletion with concurrent deletion of *PTEN* is frequently observed in metastasis, but not in primary disease (12, 13).

**INPP4B.** The inositol polyphosphate 4-phosphatase (INPP4) activity was first identified in brain lysates (28), and cloning of the human cDNA revealed that it was widely expressed and responsible for most of the cell's phosphatase activity toward the 4'-position on PIPs (29). A second human gene sharing 42% identity at the protein level was soon identified and classified as type II phosphatase (30) with the gene name *INPP4B*. *In vitro* analysis showed that the enzyme primarily hydrolyzes PI(3,4)P<sub>2</sub> at the 4-position through its C-terminal catalytic domain to generate PI(3)P (29), a result that was confirmed in cells (31). Through this action, INPP4B suppresses PI(3,4)P<sub>2</sub>, which can still serve as a platform for AKT recruitment and activation (31). In agreement, knockdown of *INPP4B* was found to phenocopy hallmarks of *PTEN* suppression, such as increased AKT signaling triggered by insulin (31, 32), and showed an increased p53-dependent cellular senescence response upon co-suppression with *PTEN*. Taken together, PTEN and INPP4B have emerged as the most strongly cooperating lipid level PI3K pathway phosphatases.

### The protein-level phosphatases

**PHLPP1.** The Pleckstrin homology domain leucine-rich repeat protein phosphatase 1 (PHLPP1) was discovered in a logical search for AKT antagonists that link a phosphatase to a Pleckstrin homology domain (33). PHLPP1, previously implicated in circadian rhythms (34), fulfilled these criteria and was confirmed to directly dephosphorylate and inactivate AKT at the serine 473 activation site (33) in addition to other targets (reviewed in ref. 35). The gene locates to chromosome 18q21 and expresses two alternatively spliced isoforms. PHLPP1b differs from PHLPP1a by a 50-kDa N-terminal extension, containing a RAS-association domain



**Figure 1.** Core phosphatases of the PI3K pathway. PTEN and INPP4B phosphatases inactivate PIP-lipid second messengers to prevent AKT activation. Functionally, they both antagonize class I PI3K-dependent membrane recruitment of AKT. PHLPP1 and PHLPP2 revert AKT activation by dephosphorylation at serine 473 to antagonize the phosphorylation that the mTOR complex 2 carries out on this site. AKT activation signals mTORC1 activation via inhibition of the TSC tumor suppressor complex. Outcome prediction: phosphatase status at the DNA, RNA, or protein level can be used to predict disease outcome.

of still unclear function. Both isoforms share the same features: the Pleckstrin homology domain, leucine-rich repeats, a PP2C catalytic domain, and a PDZ-ligand domain (reviewed in ref. 35). *PHLPP1* has been well studied in cancer models (13, 36–38), development and function of T cells (39), cardiac cell survival (40), and circadian rhythms (41). In the mouse prostate, *Phlpp1* loss leads to Akt-driven neoplasia and synergizes with partial *Pten* loss to accelerate tumor proliferation, onset, and incidence. Importantly, loss of *Phlpp1*, just like loss of *Pten*, triggers activation of p53, which causes cellular senescence. This response acts as a barrier against disease progression and is spontaneously overcome by p53 inactivation in the mutant mice (13).

**PHLPP2.** *PHLPP2* (on chromosome 16q21) shares its domain structure with the longer *PHLPP1b* splice isoform (42). Just like *PHLPP1*, *PHLPP2* is also ubiquitously expressed in most tissues and shows highest abundance in the brain. *PHLPP2* also acts predominantly on the serine 473 site of AKT and differential specificities of *PHLPP1/2* for AKT1/2/3 have been reported *in vitro* (43, 44) and are being investigated further in animal knockout models. One critical distinction between *Phlpp1* and *Phlpp2* is their differential response to

PI3K pathway activation in genetically controlled experiments: Whereas *Phlpp1* levels remain constant, *Phlpp2* is surging to antagonize Akt (13). Thus, after *Pten* loss, *Phlpp2* is critically attenuating pathway output. It remains to be seen if the two *PHLPP* isoforms show differential tissue-specific roles for this function. Mechanistically, the *PHLPP* protein levels are regulated downstream of mTORC1, thus linking levels and activity to PI3K pathway output in a negative feedback (refs. 13 and 45; reviewed in ref. 46). This role of *PHLPP2* in partially substituting for *PTEN* may explain the frequent codeletion of *PTEN* and *PHLPP2* in lethal prostate cancers.

### Clinical-Translational Advances

#### Phosphatases as prognostic biomarkers

**Single-gene associations.** *PTEN* has been extensively investigated as a biomarker for prediction of disease outcome across many cancer types (47). As a single factor, low *PTEN* gene expression is associated with prostate metastasis (48), faster rising PSA levels after surgery (13, 49), and castration resistance (50). In breast cancer, signatures of *PTEN* loss have been associated with poor prognosis (51),

similar to findings in colon cancer (52). Several studies showed the correlation between disease progression and/or relapse after intervention, when low or absent PTEN protein levels were detected in a prostate tumor (53–55). Similarly, PTEN protein status has been associated with better response to HER2 targeted therapy in breast cancer (56–59), although the straightforward correlation has been called into question by a recent large-scale study (60), which highlighted a major issue with pathway biomarkers: How much reduction of a tumor suppressor is called to be functionally relevant (see also Conclusions; ref. 61)?

Gene loss and reduction of INPP4B protein have been frequently seen in breast and ovarian cancer and correlated with increased progression of disease and shorter overall survival (31, 32). In basal-like breast cancer, furthermore, loss of *INPP4B* (and *PTEN*) strongly correlated with PI3K pathway activation as confirmed through the TCGA consortium (refs. 32 and 62; reviewed in ref. 63). INPP4B protein is also frequently lowered in prostate cancer, an event that was associated with shorter times to biochemical recurrence (ref. 64, reviewed in ref. 65). Intriguingly, the study found that the androgen receptor positively regulated INPP4B transcription and protein levels, consistent with an emerging pattern of androgen receptor–mediated suppression of AKT: Two recent studies demonstrated that androgen receptor also positively regulates PHLPP1 to suppress AKT signaling (37, 38). These findings suggest that antihormone therapy could come at the price of increased AKT activity, when INPP4B (and PHLPP1) are intact. Decreased INPP4B expression was furthermore found to correlate with disease progression in melanocytic tumors (66). Collectively, these results confirm the key pathway position of INPP4B and point to its usefulness as a pathway biomarker.

The PHLPP phosphatases have quickly moved into the spotlight of tumor suppressor studies by virtue of their ability to directly dephosphorylate AKT kinase. Strong evidence to confirm the mouse tumor-suppressive function of *Phlpp1* in humans has come from studies on prostate cancer, where the gene is frequently deleted (12). The expression analysis of clinically annotated patient samples from this study revealed significant association of low *PHLPP1/2* expression with disease recurrence after surgery (13). In colon cancer, reduced protein levels have been described (67), and PHLPP1 status has also been linked to treatment response after chemotherapy and hormone therapy (37, 38, 68). A cancer-associated polymorphism in the *PHLPP2* gene that reduces AKT-suppressing activity has been identified (69) and validated in breast and ovarian cancer (70, 71). Furthermore, several studies have shown a compensatory role for PHLPP proteins after pathway activation, for example through *PTEN* loss. This failsafe response is triggered by aberrant mTOR activation and serves to limit cell proliferation (13, 45). Thus, on the one hand, the PHLPP phosphatases are emerging as critical pathway breaks at the protein level, and, on the other hand, they serve as rheostats that actively dampen the malfunction of lipid-level phosphatases.

### Recovering phosphatase function through target therapy

At the lipid level, several candidate drugs against PI3Ks have shown success and are currently in advanced clinical trials (see Table 1, lipid kinase inhibitors). Functionally, this approach supports or recovers PTEN or INPP4B activity, or it reverts activity of mutant PI3K. The BKM120 inhibitor (buparlisib), which has activity against all four isoforms of the class I PI3K catalytic subunit (p110- $\alpha$ , - $\beta$ , - $\gamma$ , and - $\delta$ ), is currently in a phase III trial for metastatic (HR<sup>+</sup>, HER2<sup>-</sup>) breast cancer. Other approaches include isoform-specific inhibitors, such as CAL101 (idelalisib), a p110- $\delta$  inhibitor, for which phase III trials showed a significant improvement in overall survival for chronic lymphocytic leukemia (72); in this type of cancer PTEN LOH has been observed at 20% frequency (73).

The PI3K pathway has been successfully targeted at the protein level after the discovery of the naturally occurring mTORC1 inhibitor rapamycin and its derivatives, the rapalogs. They are used as immunosuppressants after organ transplantation as they inhibit T-cell activation (74). The RAD001 derivative (everolimus) has been approved by the U.S. Food and Drug Administration (FDA) in 2010 for the treatment of tuberous sclerosis (TSC) syndrome, which predisposes patients with inherited TSC mutations (see Table 1) to precancerous lesions. In cancer, the drug has been approved for advanced kidney cancer, a TSC-associated astrocytoma, an HR<sup>+</sup>, HER2<sup>-</sup> breast cancer subtype, and for treatment of pancreatic neuroendocrine tumors (PNET; reviewed in ref. 75). AKT inhibitors are targeting the protein by two mechanisms. Allosteric inhibitors, such as MK2206 or Perifosine prevent translocation of AKT to the plasma membrane, thus it cannot be activated by phosphorylation. The ATP-competitive inhibitor (e.g., GSK2110183, Afuresertib) in contrast targets the AKT active site, which results in hyperphosphorylation of the kinase. Both MK2206 and GSK2110183 are currently in phase II trials against blood and solid cancers (see Table 1), whereas the phase III trials of perifosine in colon cancer and multiple myeloma have not shown significant results.

Finally, several promising compounds exhibit so-called dual specificity, because of the close evolutionary relationship between the kinase domains of PI3Ks and mTOR—in spite of the diversification into lipid-specific and protein-specific kinases (76). Although no dual specificity inhibitor has so far been FDA approved, several of them are currently being evaluated in phase II trials (see Table 1) against PNET and other advanced cancers.

### Conclusions

The lipid-level and protein-level phosphatases of the PI3K pathway form a tight natural network against cancer, which should be routinely monitored at the genetic and protein level to assist in outcome prediction. In addition, successful drug discovery programs have afforded us with many compounds that can support or replace core functions of these phosphatases when they are lost. The

**Table 1.** Clinical trial relevance

Kinase target(s)	Antagonistic phosphatase	Drug	Trial phase	Cancer	Trial ID
Protein kinase inhibitors					
AKT		GSK2110183	Phase II	Solid tumors, hematologic malignancies	NCT01531894
AKT		MK2206	Phase II	Relapsed or refractory acute myeloid leukemia	NCT01253447
mTORC1		Everolimus	Phase II	Melanoma	NCT01960829
mTORC1		Sirolimus	Phase II	Hepatocellular carcinoma	NCT01374750
mTORC1		Temsirolimus	Phase I/II	Advanced cancers	NCT00877773
mTORC1/2		INK128	Phase I	Advanced nonhematologic malignancies	NCT01899053
mTORC1/2	PHLPP1/2	OSI-027	Phase I	Solid tumor, lymphoma	NCT00698253
mTORC1/2		AZD8055	Phase I	Glioblastoma multiforme, other brain tumors	NCT01316809
Lipid kinase inhibitors					
PI3K		BAY80-6946	Phase II	Non-Hodgkin lymphoma	NCT01660451
PI3K		BKM120	Phase III	Metastatic breast cancer HR <sup>+</sup> , HER2 <sup>-</sup>	NCT01633060
PI3K	PTEN	CAL101	Phase III	Chronic lymphocytic leukemia	NCT01659021
PI3K		GDC0941	Phase II	Non-small cell lung cancer	NCT01493843
PI3K		IPI145	Phase II	Indolent non-Hodgkin lymphoma	NCT01882803
PI3K		XL147	Phase I/II	Breast cancer, breast neoplasms	NCT01042925
Dual specificity inhibitors					
PI3K mTORC1/2		BEZ235	Phase II	Pancreatic neuroendocrine tumors (pNET)	NCT01628913
PI3K mTORC1/2	PTEN	BGT226	Phase I/II	Advanced breast cancer	NCT00600275
PI3K mTORC1/2		PF04691502	Phase II	Endometrial neoplasms	NCT01420081
PI3K mTORC1/2	PHLPP1/2	PF05212384	Phase II	Metastatic colorectal cancer	NCT01925274
PI3K mTORC1/2		XL765	Phase I	Glioblastoma, astrocytoma	NCT01240460

NOTE: Phosphatase status could be used for patient stratification into matching clinical trials. Current phase II/III trials against pathway targets at the lipid and/or protein level are listed. Phosphatases that are functionally supported by each approach are indicated in the relevant phosphatase column.

challenge now consists in matching molecular genetic events on the phosphatase side with therapeutic strategies on the inhibitor side. It remains unclear if alterations at a specific level of the pathway sensitize cancers to drugs that inhibit at the same exact level. For example, one could envision *PTEN*-mutant tumors to be more sensitive to PI3K than Akt or mTORC inhibitors. Such linkages can be established in controlled, primary model systems using pharmacologic and genetic tools. However, it is also expected that these linkages are perturbed by the context of spontaneous aberrations and feedbacks that arise in a tumor (77). Furthermore, functional readouts for the relevance of phosphatase alterations are needed to prevent false status calls, as recently suggested by comparing different studies on *PTEN* status in trastuzumab therapy of breast cancer (reviewed in ref. 61). Yet, in spite of this

complexity, there is hope for discovering distinct linkages between alterations and therapeutic effects. Patients harboring *TSC* germline mutations that activate mTORC1 clearly benefit from targeting of mTORC1 with rapalogs. Thus, it can be envisioned that the precise matching of drugs with predetermined pathway defects and known resistance routes of a tumor may provide a winning anticancer strategy.

#### Authors' Contributions

**Conception and design:** M. Chen, D.G. Nowak, L.C. Trotman  
**Writing, review, and/or revision of the manuscript:** M. Chen, D.G. Nowak, L.C. Trotman

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