A CERTain Role for Ceramide in Taxane-Induced Cell Death

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An unexpected benefit of functional genomic screens is that at times they answer questions that they were not designed to ask. A siRNA screen reported by Swanton et al. in this issue of *Cancer Cell* reveals that silencing of spindle assembly checkpoint genes facilitates mitotic slippage, resulting in escape from taxane-induced cell death, aneuploidy, and chromosomal instability, hallmarks of taxane resistance. Unexpectedly, the screen disclosed that the sphingolipid ceramide is a key regulator of the taxane-mediated spindle assembly checkpoint and taxane-induced cell death. Ceramide metabolism thus serves as a legitimate target for modulation of taxane effect on tumors.

An extensive literature indicates that the taxane group of anticancer drugs, which bind mitotic spindle elements, must provoke mitotic arrest to induce cell kill via apoptosis. Mitotic-phase cell-cycle arrest is normally regulated by spindle assembly checkpoint proteins, mostly kinases, which are activated by microtubule-unattached kinetochores or by lack of sister centromere tension and are silenced upon correct alignment of chromosomes along the mitotic spindle. This function prevents premature advance to anaphase and chromosome missegregation and protects progeny cells from aneuploidy and chromosomal instability (CIN) (Kops et al., 2005). Taxanes bind microtubules with high affinity, altering microtubule dynamics, thus aberrantly affecting tension at kinetochores that triggers sustained mitotic arrest, eventually resulting in apoptosis (Jordan and Wilson, 2004). A corollary of this model is that mitotic slippage would be anticipated to prevent taxane-induced cell death. Employing a siRNA screen designed to inactivate all human kinases and a set of associated proteins, Swanton et al. (2007) confirm that knockdown of select spindle assembly checkpoint genes suppresses paclitaxel-induced mitotic block and enables mitotic slippage, which results in reduced sensitivity to taxane lethality. While it has been generally believed that mutations in mitotic checkpoint genes are by themselves not sufficient to induce aneuploidization, requiring altered transcriptional regulation by tumor suppressors or oncogene products (Kops et al., 2005), Swanton et al. report that knockdown of mitotic spindle checkpoint genes is sufficient to cause polyploidy and anomalous centrosome numbers, even in the absence of taxane exposure. This unexpected finding establishes a firm linkage between silencing of specific mitotic checkpoint genes, mitotic slippage, aneuploidy, and CIN, hallmarks of the taxane-resistant phenotype. Consistent with this notion, previous data indicate that tumor cells with CIN do not typically arrest at mitosis after taxane treatment, nor do they respond well to these drugs in the clinic (Roberts et al., 1990).

Another unexpected outcome of the screen was the discovery that genes associated with metabolism of the sphingolipid ceramide regulate the taxane-mediated spindle assembly checkpoint and taxane-induced cell death. siRNA inactivation of *COL4A3BP*, the gene encoding the ceramide transfer protein CERT (Hanada et al., 2007), provided a significant increase in paclitaxel chemosensitivity. CERT normally extracts newly synthesized ceramide from ER membranes and traffics it in a nonvesicular manner to the Golgi apparatus for sphingomyelin synthesis. CERT contains several functional domains that participate in this action, including a START domain that binds ceramide, a pleckstrin homology domain that recognizes Golgi phosphatidylinositol 4-monophosphate, and a FFAT motif that interacts with the ER resident protein VAP. CERT was recently cloned using LY-A cells that display defective sphingomyelin synthesis due to markedly diminished transfer of ER ceramide to Golgi. Overexpressing CERT reversed the genetic defects. Consistent with these observations, inactivation of CERT by a point mutation in *COL4A3BP* or use of the stereospecific CERT inhibitor (1*R*,3*R*)*N*-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)dodecamide (HPA-12) caused an increase in ER ceramide content.

Relative to taxane sensitivity, the studies of Swanton et al. show that LY-A cells are more sensitive to paclitaxel than LY-A cells overexpressing CERT, while siRNA to *COL4A3BP* sensitizes LY-A/hCERT cells to paclitaxel. Furthermore, drug-resistant ADR/RES breast and SKOV3-TR ovarian cancer cells were found to have high levels of CERT, and siRNA to *COL4A3BP* sensitized ADR/RES cells to paclitaxel. Conversely, lowering ceramide produced a phenotype opposite that of *COL4A3BP* inactivation. siRNA inactivation of *GBA*,

the gene encoding β-glucosidase that converts glucosylceramide to ceramide, which presumably lowers ceramide levels, reduced mitotic checkpoint activity, increased drug resistance, and enhanced aneuploidy. Finally, data from 14 evaluable patients receiving neoadjuvant paclitaxel for ovarian carcinoma revealed increased *COL4A3BP* expression in patients failing treatment, strongly implying direct involvement of CERT in paclitaxel-mediated cell kill. Epistasis analysis of these data taken together indicates that the mitotic checkpoint likely functions upstream of (or parallel to) ceramide (Figure 1).

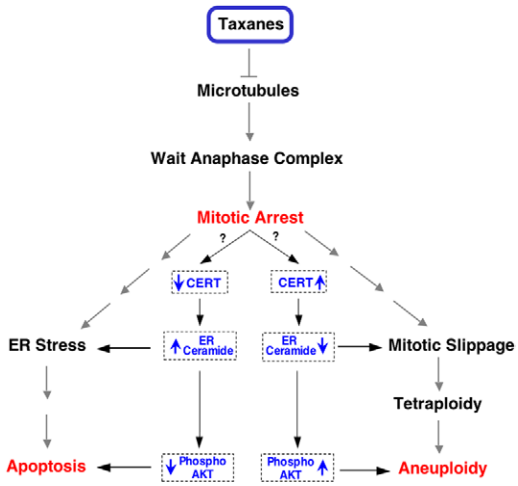

Figure 1. A Proposed Model Defining the Role of Ceramide in the Response of Cancer Cells to Taxanes

Binding of taxanes to microtubules aberrantly engages the mitotic checkpoint, effecting sustained mitotic arrest. The two outcomes of sustained mitotic arrest, apoptosis or mitotic slippage, are regulated by the level of ceramide, and its downstream effector, Akt. While ceramide elevation is a known component of taxane-mediated death, the studies by Swanton et al. indicate an ER topology for the taxane-induced ceramide elevation. Sustained ER ceramide elevation coordinately activates the ER stress response and inactivates antiapoptotic Akt, thus leading to apoptosis. Conversely, mitotic arrest in the absence of ER ceramide elevation shifts the balance toward mitotic slippage, with progression to aneuploidy requiring activated Akt. Additional studies will be required to formally document that the ER is the site of the ceramide increase, and the role of CERT in this process.

COL4A3BP siRNA silencing in paclitaxel-treated cells also reduced cell number and synergistically increased caspase 3/7 activity, suggesting that ceramide acts to enhance taxane-induced apoptosis. These observations are consistent with multiple recent reports indicating that taxane lethality is associated with increased cellular ceramide, while ceramide inhibition confers taxane resistance (Asakuma et al., 2003; Prinetti et al., 2006). Based on the ER topology of CERT function, Swanton et al. focused on ER stress as a source of signals for paclitaxel-induced apoptotic death. The ER stress response (also known as the unfolded protein response) is a conscripted sequence of events that lead to adaptation or apoptosis (Boyce and Yuan, 2006; Szegezdi et al., 2006). The ER is highly sensitive to stresses that perturb energy levels, Ca^{2+} metabolism, or the redox state. These stresses reduce ER capacity for protein folding, resulting in accumulation and aggregation of unfolded proteins. Unfolded proteins shift the ER chaperone Grp78 from three receptors, the protein kinase PERK, the transcription factor ATF6, and the dual activity serine/threonine kinase and endonuclease IRE1. Sequential activation of these receptors either restores homeostasis, or dependent on the extent of damage, promotes apoptosis, in part through ATF-4-mediated induction of CHOP. Although the studies by Swanton et al. do not examine the ER stress response in detail, they identify taxanes as stimulators of PERK autophosphorylation, followed by induction of proapoptotic CHOP. These data are consistent with prior studies showing a correlation between CHOP mRNA increases and the response of primary breast cancer to paclitaxel (de las Alas et al., 2000). Given that *COL4A3BP* inactivation increased caspase activity after treatment of HCT-116 cells with the ER stressor tunicamycin, follow-up studies examined the interaction between paclitaxel and *COL4A3BP* inactivation on ER stress. While siRNA to *COL4A3BP* or CERT inactivation using HPA-12 induced phosphoPERK to the same extent as paclitaxel, the combined effect of CERT inactivation and paclitaxel was synergistic. These studies define for the first time ceramide as a biochemical mediator of ER stress and, coupled with the epistasis data regarding the mitotic checkpoint, suggest that ceramide may represent a molecular switch between apoptosis and mitotic slippage (Figure 1).

How ceramide might discriminate between apoptosis and mitotic slippage can only be speculated. The Swanton et al. studies define Akt kinase as candidate mediator of apoptosis/mitotic slippage. Data in the literature indicate that phosphorylation of Akt on Ser473 is activating, yielding antiapoptosis signaling in multiple systems, while dephosphorylation of this site is generally proapoptotic (Brazil et al., 2004). Further, Jin and Woodgett (2005) provided evidence that active Akt may play a role in aneuploidy and multinucleation. Swanton et al. report that paclitaxel reduces Akt phosphorylation on Ser473 and Thr308 in cells destined to die by apoptosis, while dephosphorylation was absent upon siRNA inactivation of spindle assembly checkpoint genes. Manipulation of genes that regulate ceramide metabolism yielded complementary phenotypes. *COL4A3BP* inactivation, which enhances paclitaxel-induced apoptosis, enhanced paclitaxel-induced Akt dephosphorylation, while GBA inactivation, which confers mitotic slippage, prevented dephosphorylation. These data are consistent with a recent publication by Asakuma et al. (2003) showing in renal cancer cells that paclitaxel reduces phosphoAkt(Ser473) via ceramide generation, amplifying paclitaxel-induced apoptosis and increasing TRAIL-induced death. How ER ceramide levels might modulate the phosphorylation status and compartmentalization of Akt at the plasma membrane has not been explored, although ceramide is known to regulate a set of kinases (KSR, PKCζ) and phosphatases (CAPP) that might transmit such signals (Kolesnick, 2002; Ogretmen and Hannun, 2004). Further, although a number of context-dependent media-

tors (c-Jun kinase, ASK1, eIF2α) have been implicated in linking ER stress to the apoptotic machinery, no coherent picture has emerged, making it difficult to currently speculate as to the connection between ceramide signaling, Akt activation/inactivation, and these proapoptotic mediators (Boyce and Yuan, 2006).

In sum, the studies by Swanton et al. provide extensive new evidence, gleaned from diverse experimental strategies, that CERT, and its client ceramide, are integral to paclitaxel-mediated cell death. Further, these studies identify ER stress as a previously unrecognized source of signals leading to apoptotic cell death upon taxane exposure. A challenge posed by these studies is to identify which of the many biochemical events likely to be dysregulated by prolonged mitotic checkpoint activity yields the ER stress response. An additional challenge is to define the mechanism by which ER ceramide might regulate taxane sensitivity biochemically and/or pharmacologically. While inhibition of glucosidase attenuated the spindle checkpoint by conferring mitotic slippage, inserting sphingolipid metabolism into this process for the first time, there is insufficient data presented here to ascribe such regulation to ER ceramide levels. Although these studies probably bring up more questions than they answer, they do provide unequivocal data that signaling associated with ceramide metabolism regulates taxane-induced apoptosis, the fundamental event in taxane-mediated tumor response.

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Chromatin Modulation by Oncogenic Transcription Factors: New Complexity, New Therapeutic Targets

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Oncogenic transcription factors such as PML-RARα, RUNX1-MTG8, and others work in large part by the recruitment of inhibitors of gene transcription to target promoters leading to aberrant repression of gene expression. PML-RARα, an archetypal chimeric oncoprotein, was previously shown to bring complexes of histone deacetylases (HDACs), histone methyltransferases (HMTases), and DNA methyl transferases (DNMTs) to target genes. In this issue of *Cancer Cell*, Villa et al. show that the full complement of chromatin machinery can be commandeered by these transcription factors with the polycomb group of proteins representing the newest identified recruit.

The Polycomb Group (PcG) of proteins were initially discovered in *Drosophila* as epigenetic silencers of homeotic (HOX) genes. PcG proteins have since been shown to be required for the X chromosome inactivation, germline development, stem cell renewal, hematopoiesis, and cell proliferation.