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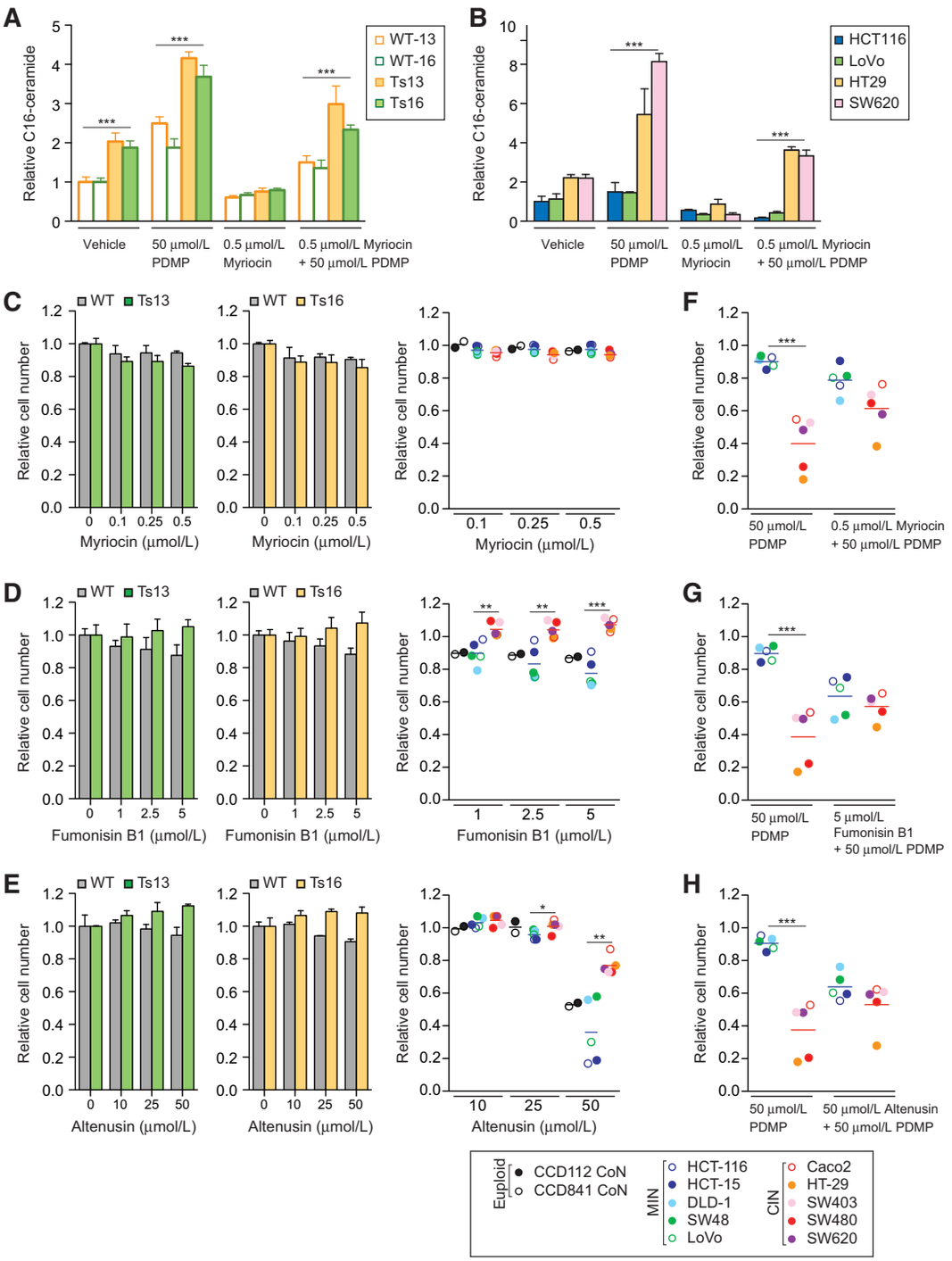

Figure 6.

Effects of altering sphingolipid metabolism on DL-PDMP sensitivity in aneuploid cells. **A,** Mass spectrometry–based quantification of C16-ceramide levels in wild-type, trisomy 13, and trisomy 16 cells following a 24-hour treatment with the indicated compounds. Ceramide levels were normalized to those of wild-type (WT) cells treated with vehicle only, which was set to 1. **B,** Mass spectrometry–based quantification of C16-ceramide levels in MIN and CIN cancer cell lines following a 24-hour treatment with the indicated compounds. Ceramide levels were normalized to those of HCT116 cells treated with vehicle only, which was set to 1. **C–E,** Wild-type and trisomic cells (left and middle) and human colorectal cancer cells (right) were treated with the indicated concentrations of myriocin (**C**), fumonisin B1 (**D**), or altenusin (**E**). Cell number was determined after 72 hours. Cell number of drug-treated cells was normalized to that of the same cell line treated with vehicle only. **F–H,** MIN and CIN cancer cell lines were treated with 50 μmol/L DL-PDMP either alone or in combination with myriocin (0.5 μmol/L; **F**), fumonisin B1 (5 μmol/L; **G**), or altenusin (50 μmol/L; **H**). Cell number was determined after 72 hours. Cell number of drug treated cells was normalized to that of the same cell line treated with vehicle only. The data are shown as the mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, *t* test.

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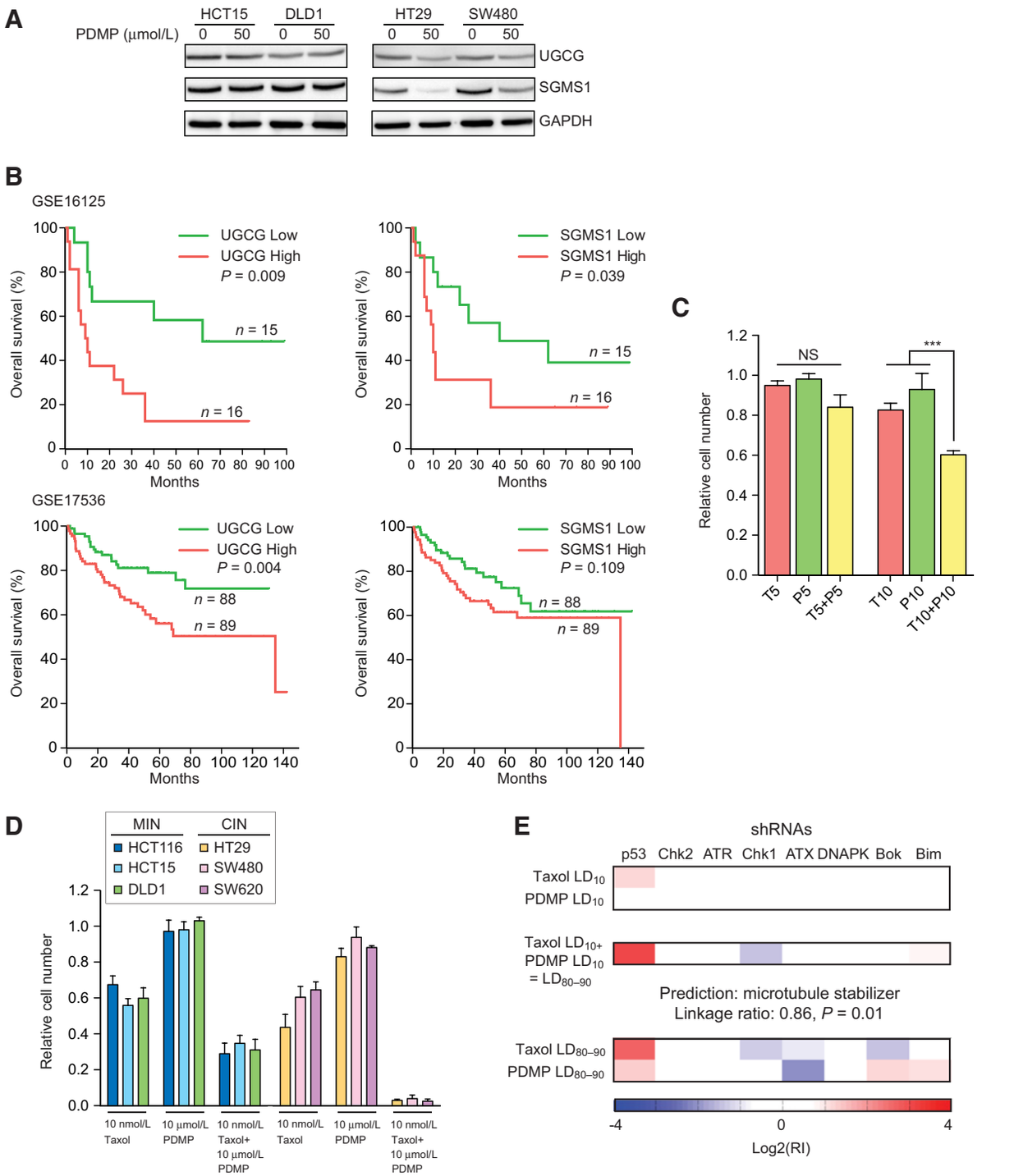

Figure 7.

Effects of altering ceramide levels on cancer development and treatment. **A,** MIN and CIN colorectal cancer cells were treated with DL-PDMP at the indicated doses for 24 hours and UGCG and SGMS1 levels were determined by immunoblot analysis. GAPDH served as a loading control. **B,** Survival analysis (log-rank test) of colon cancer patients expressing high or low levels of *UGCG* (left) or *SGMS1* (right). Dataset GSE16125 is shown on the top; bottom, data set GSE17536. **C** and **D,** Wild-type MEFs (**C**) or MIN and CIN colorectal cancer cell lines (**D**) were treated with the indicated concentrations of DL-PDMP (P) and Taxol (T). Cell number was determined after 72 hours. Cell number of drug–treated cells was normalized to that of cells treated with vehicle only. **E,** *Eμ-Mycp*$^{19arf-/-}$ lymphoma cells were treated with Taxol and DL-PDMP. The shRNA signatures generated at LD10 single drug doses, which produce $LD_{80–90}$ signatures when combined, are shown at the top. The signature that is produced when the two drugs are combined to yield an LD of 80–90 is shown in the middle panel. $LD_{80–90}$ signatures for the single drugs are shown at the bottom. The data are shown as the mean ± SD. ***, $P < 0.001$, *t* test. NS, nonsignificant.

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reason for the increased sensitivity of CIN colorectal cancer cells to compounds that elevate intracellular ceramide levels. We note however that changes in protein levels of these enzymes do not appear to be altered in untreated cells, indicating that other mechanisms must be responsible for increased basal ceramide levels in CIN colorectal cancer cell lines.

An alternative way to assess the importance of ceramide-metabolizing enzymes in colorectal cancer cell proliferation is to ask whether expression of these enzymes predicts patient survival. Specifically, upregulation of *UGCG* and *SGMS1* ought to cause a decrease in intracellular ceramide levels and hence increased tumor growth, which would lead to decreased survival. To test this possibility, we correlated expression of *UGCG* and *SGMS1* with survival in two colorectal cancer studies (37, 38). Analyses of clinical datasets GSE16125 and GSE17536 revealed that high levels of *UGCG* and to some extent *SGMS1* are associated with decreased survival (Fig. 7B). This observation raises the possibility that upregulation of either or both genes could promote the survival of aneuploid cancer cells and thus affect morbidity. We further note that targeting either or both enzymes may have a therapeutic benefit.

DL-PDMP enhances the cytotoxic effects of the aneuploidy-inducing chemotherapeutic Taxol

Taxol is the most commonly used chemotherapeutic agent in the treatment of both solid tumors and hematopoietic malignancies (39). Taxol is a microtubule poison that arrests cells in mitosis, but induces chromosome mis-segregation at clinically relevant concentrations (40). If DL-PDMP indeed preferentially induces apoptosis in aneuploid cells, we predict that this ceramide analogue ought to enhance the cytotoxic effects of Taxol.

We treated wild-type MEFs with low concentrations of Taxol, DL-PDMP, or a combination thereof, and found DL-PDMP to subtly enhance the toxicity of Taxol (Fig. 7C). The effects of combining Taxol and DL-PDMP were more striking in MIN colorectal cancer cell lines and especially evident in CIN colorectal cancer cell lines (Fig. 7D).

To further characterize the enhancement of Taxol's cytotoxicity by DL-PDMP, we employed a previously developed method to examine anticancer drug combination synergy and mechanism of action (23, 41). In this assay, murine $E\mu$-$Myc^{p19arf-/-}$ lymphoma cells harboring eight different shRNA-GFP constructs are treated at equipotent doses with either a single compound or compound combinations. The pattern of resistance and sensitivity of these shRNA-harboring cells to a drug or drug combination is then used to bioinformatically classify the mechanism of action of the drugs being tested.

For this classification, we first needed to determine the drug concentrations required to kill 80%–90% (LD_{80-90}) of the lymphoma cells. We found that the LD80-90 of DL-PDMP was between 61 μmol/L and 85 μmol/L. The same degree of growth inhibition was accomplished at Taxol concentrations of 17 nmol/L to 20 nmol/L. Notably, the LD_{80-90} of the DL-PDMP and Taxol combination was achieved at concentrations of 7–14 μmol/L DL-PDMP and 5–6.25 nmol/L Taxol. When applied individually at these concentrations, the compounds reduced proliferation by only 10%. The combination of DL-PDMP and Taxol was the most synergistic combination tested in this system relative to the Bliss Independence additivity model (41).

To characterize the mechanism of action of the synergistic DL-PDMP/Taxol combination, we obtained the eight shRNA signature of cells treated with either compound alone or in combination. As expected, Taxol classified as a microtubule stabilizer signature (Taxol LD_{80-90}; Fig. 7E). This signature is characterized by increased resistance to the drug upon p53 knockdown and increased sensitivity upon knockdown of Chk1 and Bok (Fig. 7E; ref. 41). DL-PDMP classified as a new previously uncharacterized mechanism of action, as judged by its eight shRNA signature. This novel signature is characterized by increased sensitivity to DL-PDMP upon knockdown of the PI3K-like ATX (Fig. 7E). The signature of the combination of DL-PDMP and Taxol classified as a microtubule stabilizer indicating that DL-PDMP enhances the effects of Taxol. Our results raise the exciting possibility that compounds that like DL-PDMP lead to an increase in intracellular ceramide levels could significantly improve the efficacy of Taxane-based chemotherapeutics.

Discussion

Previous studies have identified both gene-specific effects of aneuploidy as well as phenotypes seen in many different aneuploid cells (42–44). The latter category includes proliferation defects, proteotoxic and energy stress, as well as genomic instability. Here, we describe another broadly observed aneuploidy-associated phenotype—dysregulation of sphingolipid metabolism that results in elevated levels of ceramide. An unbiased chemical genetic approach identified two compounds that increase intracellular ceramide levels, DL-PDMP and C8-ceramide, to induce apoptosis in aneuploid primary and cancer cell lines. Our data further indicate that one reason for this increased sensitivity is that aneuploid cells already harbor elevated levels of this proapoptotic lipid. Importantly, the adverse effects of ceramide on the proliferation of aneuploid cells appear conserved across species. In an unbiased genetic selection, Torres and coworkers identified mutations that cause a decrease in intracellular ceramide levels to improve the fitness of aneuploidy-budding yeast cells (ref. 45; E.M. Torres, personal communication). Thus, two nonhypothesis-driven approaches in different experimental systems have uncovered the same biological process as being deregulated and causing a fitness defect in aneuploid cells. These observations lend support to the idea that large-scale changes in a cell's karyotype and hence proteome impact cell physiology in fundamentally the same way in all eukaryotes.

A key question posed by our results is why ceramide levels are elevated in aneuploid cells. We find that inhibition of all three ceramide synthesis pathways, the *de novo* synthesis pathway, the salvage pathway and the sphingomyelin hydrolysis pathway, are required for DL-PDMP–induced apoptosis in aneuploid cells. This finding indicates that all three pathways contribute to high levels of ceramide in aneuploid cells. However, the fact that chemical inhibition of the salvage pathway and the sphingomyelin hydrolysis pathway, but not of the *de novo* synthesis pathway improves the proliferation of trisomic cells, suggests that these two pathways are particularly important for the accumulation of ceramides in aneuploid cells.

Why baseline ceramide levels are higher in aneuploid cell lines remains unknown. The abundance of enzymes involved in the biosynthesis and metabolism of ceramides is not noticeably altered in aneuploid cells. This finding indicates that enzyme activities rather than levels are altered in aneuploid cells. Consistent with this conclusion are previous studies that showed DNA damage stress to cause ceramide accumulation by affecting the

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activity of ceramide-producing and -metabolizing enzymes. In HeLa and U937 cells, UV irradiation triggers the hydrolysis of sphingomyelin by acid sphingomyelinase thereby raising intracellular ceramide levels (46). The DNA-damaging agent daunorubicin has been shown to stimulate *de novo* synthesis of ceramide via activation of ceramide synthase (47). Aneuploid cells experience a number of stresses including DNA damage (8). We propose that it is these aneuploidy-associated stresses that alter the activity of ceramide biosynthesis and metabolizing enzymes thereby causing an increase in intracellular ceramide levels. When aneuploid cells are treated with DL-PDMP or C8-ceramide, the ceramides accumulate to even higher levels and induce apoptosis by previously described mechanisms (48). It is also possible that DL-PDMP induces yet further alterations in ceramide metabolism in aneuploid cells. In this regard, we note that levels of two enzymes critical for the metabolism of ceramides into other lipids, UGCG and SGMS1, do decrease upon treatment of CIN colorectal cancer cells with DL-PDMP.

Our results indicate that aneuploid primary cells are more sensitive to increased ceramide levels than euploid cells. A similar differential sensitivity is seen in colorectal cancer. Highly aneuploid CIN cancer cell lines were more sensitive to DL-PDMP and C8-ceramide than pseudo-diploid MIN cancer cell lines. This differential sensitivity of aneuploid and pseudo-diploid cell lines does, however, not exist in all cancer types. Myeloid leukemia cell lines are very sensitive to DL-PDMP treatment irrespective of whether or not they are aneuploid. The absence of a differential effect in this cancer may be due to the fact that all myeloid leukemias are sensitive to ceramides for additional reasons. Metabolizing ceramide into other lipids is central for myeloid leukemia cells to proliferate (49). Consistent with this finding, SGMS1 activity has been shown to be upregulated by the *BCR-ABL* oncogene, the cause of CML (50). In the AML cell line HL-60, SGMS1 activity is also upregulated (50). Finally, mutations in the neutral sphingomyelinase (nSMase) gene *SMPD3*, which encodes a primary ceramide biosynthesis enzyme, were identified in 5 of 92 AMLs and 8 of 131 acute lymphoid leukemias (ALL), but not in other tumor types (51). Together, our findings and that of others suggest that aneuploidy sensitizes some cells types to increasing intracellular ceramide levels. In other cell types, ceramide sensitivity may already be so high that karyotype no longer matters.

Ceramide biosynthesis regulation has been shown to be clinically relevant in cancer. The first report of a role of sphingolipid metabolism in tumorigenesis was published more than 30 years ago (52). Since then it has become clear that this lipid synthesis pathway contributes to disease progression and outcome in many different cancers. In human gliomas, low levels of ceramide are associated with poor outcome (53). A similar correlation was described in ovarian cancer. In this tumor type, ceramide levels are reduced compared with normal ovarian tissues and tumors harbor biologically inactive dihydroceramide instead (54). Our analyses of two colorectal cancer datasets also support the idea that low levels of ceramide are associated with aggressive disease.

Targeting sphingolipid metabolism has been explored in a variety of clinical contexts including obesity, type II diabetes, Gaucher disease, and cancer (55). In the case of glioblastoma, for example, increasing ceramide levels has been suggested as a therapy following relapse after chemo- or radiotherapy. Recurrent glioblastomas harbor high levels of sphingosine kinase activity. Targeting sphingosine kinase (SK) would prevent conversion of ceramide into sphingosine 1-phosphate (S1P) hence causing an increase in ceramide levels and apoptosis (22). Our study suggests that the aneuploid state causes an increase in intracellular ceramide levels. Given that most solid human tumors are aneuploid, treatments that elevate intracellular ceramide to levels where the lipid induces apoptosis could represent a new cancer therapy with ideal properties—broad-spectrum efficacy and selectivity. To explore this avenue, it is important to understand how DL-PDMP increases intracellular ceramide levels. DL-PDMP is a ceramide analogue that is thought to inhibit glucosylceramide synthase (16) thereby preventing the conversion of ceramide into glucosylceramide. However, DL-PDMP may also interfere with the function of other ceramide-metabolizing enzymes: DL-PDMP has been suggested to partially inhibit SGMS as well (56). It has not escaped our attention that DL-PDMP potentially targeting multiple enzymes would make the development of high-affinity inhibitors with DL-PDMP's properties challenging.

We are nevertheless intrigued by the possibility that increasing intracellular ceramide levels synergizes with the chemotherapeutic Taxol, which at clinically relevant concentrations causes chromosome mis-segregation (40). The effects of combining Taxol with DL-PDMP are not uniform across cell lines. We find that combining Taxol and DL-PDMP dramatically reduces the effective dose of Taxol in CIN cell lines and $E\mu\text{-}Myc^{p19arf-/-}$ lymphoma cells, but augmentation was modest in MEFs and MIN cell lines. Why the response varies between cell types is not yet clear. We speculate that degree of aneuploidy could at least in part dictate sensitivity to combination treatment. Recall, primary cell lines in which only a fraction of cells are aneuploid ($BUB1b^{H/H}$ and $CDC20^{AAA}$ MEFs) show limited sensitivity to DL-PDMP presumably because a good fraction of cells in the population is euploid. A similar logic could apply to euploid or pseudodiploid cells treated with low doses of Taxol. Low doses of Taxol cause aneuploidy in only a fraction of cells, limiting the impact of DL-PDMP treatment.

In closing, we note that reduction in effective dose is not only observed when Taxol was combined with DL-PDMP but also when Taxol is combined with C8-ceramide. This finding indicates that it is indeed elevated levels of ceramide that make Taxol more cytotoxic. This observation raises the exciting possibility that combining compounds that increase the intracellular ceramide levels with Taxane-based chemotherapeutics could not only create highly effective treatment regimens but may also serve as an excellent way to mitigate the serious side effects associated with taxane-based chemotherapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y.-C. Tang, M.T. Hemann, A. Amon

Development of methodology: Y.-C. Tang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-C. Tang, H. Yuwen, K. Wang, P.M. Bruno, K. Bullock, A.A. Deik, S. Santaguida, M. Trakala, S.J. Pfau, N. Zhong, L. Wang, C.B. Clish

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-C. Tang, H. Yuwen, K. Wang, P.M. Bruno, K. Bullock, A.A. Deik, S. Santaguida, M. Trakala, N. Zhong, T. Huang, C.B. Clish, M.T. Hemann, A. Amon

Writing, review, and/or revision of the manuscript: Y.-C. Tang, P.M. Bruno, C.B. Clish, A. Amon

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):Y.-C. Tang
Study supervision: Y.-C. Tang, A. Amon

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Aneuploid Cell Survival Relies upon Sphingolipid Homeostasis

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