Overexpression of Sphingosine-1-Phosphate Lyase or Inhibition of Sphingosine Kinase in Dictyostelium discoideum Results in a Selective Increase in Sensitivity to Platinum-Based Chemotherapy Drugs

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The efficacy of the chemotherapy drug cisplatin is often limited due to resistance of the tumors to the drug, and increasing the potency of cisplatin without increasing its concentration could prove beneficial. A previously characterized Dictyostelium discoideum mutant with increased resistance to cisplatin was defective in the gene encoding sphingosine-1-phosphate (S-1-P) lyase, which catalyzes the breakdown of S-1-P, an important regulatory molecule in cell function and development and in the regulation of cell fate. We hypothesized that the increased resistance to cisplatin was due to an elevation of S-1-P and predicted that lowering levels of S-1-P should increase sensitivity to the drug. We generated three strains that stably overexpress different levels of the S-1-P lyase. The overexpressor strains have reduced growth rate and, confirming the hypothesis, showed an expression-dependent increase in sensitivity to cisplatin. Consistently, treating the cells with d-erythro-N,N-dimethylsphingosine, a known inhibitor of sphingosine kinase, increased the sensitivity of mutant and parent cells to cisplatin, while addition of exogenous S-1-P or 8-Br-cyclic AMP made the cells more resistant to cisplatin. The increased sensitivity of the overexpressors to cisplatin was also observed with the cisplatin analog carboplatin. In contrast, the response to doxorubicin, 5-flurouracil, or etoposide was unaffected, indicating that the involvement of the sphingolipid metabolic pathway in modulating the response to cisplatin is not part of a global genotoxic stress response. The augmented sensitivity to cisplatin appears to be the result of an intracellular signaling function of S-1-P, because D. discoideum does not appear to have endothelial differentiation growth (EDG/S1P) receptors. Overall, the results show that modulation of the sphingolipid pathway at multiple points can result in increased sensitivity to cisplatin and has the potential for increasing the clinical usefulness of this important drug.

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that manipulations of these sphingolipid biosynthesis and bio-
degradation pathways could augment treatment with cisplatin.
Additionally, the sglAOE mutants have an impaired growth
phenotype, which results in slower proliferation in liquid cul-
tures and smaller plaque size when grown on bacteria, con-
firming the pleiotropic role of S-1-P in the regulation of cel-
lar processes.

MATERIALS AND METHODS

Strains and culture conditions. Ax3-ORF2, the parent strain for the overex-
pressing S-1-P lyase gene, and the transformation vector pDXA3C were gifts
from D. Manstein, National Institute for Medical Health, London, United King-
dom (18, 26). The 7.7-kb transformation construct carrying the myc-tagged sglA
gene (pDXA3CsoglA) is pJMS. The S-1-P lyase over expressors [sglA-mycneo5]-
[sglA-mycneo5]-2, and [sglA-mycneo5]-3 (strains SA901 to SA903) are referred to
in the text as sglAOE-1, -2, and -3. Strain SA55 contains a homologous deletion
of the sglA gene, is blasticidin resistant, and is referred to in the text as sglAΔ
(previously named cis2B [19, 20]).

Strains were stored either frozen in liquid nitrogen in a mixture of 5% di-
methyl sulfoxide (DMSO) in horse serum or as desiccated spores on silica gel at
4°C. Fresh cultures were started monthly from stocks. Cells were grown in HL5
medium (35). Clonal isolation of strains and some growth rate experiments were
performed by growing cells on SM agar in association with Klebsiella aerogenes as
a food source (43). All cell growth was done at 22°C.

Construction of sglA overexpression vector. A full-length cDNA for the S-1-P
lyase gene (sglA; accession no. [AY263052]) was derived by reverse transcrip-
tion–PCR (Gibco Superscript Preamplification System; Gibco-BRL, Gaithersburg,
Md.) by using total RNA from D. discoideum strain Ax4. First-strand synthesis
was done using the gene-specific reverse primer 5′-CTCCAGTGTCATGTGAA
GGTGTGATGGAAGGG 3′, and the ensuing PCRs were carried out using the
forward primer 5′-CTCCAGTGTCATGTGAAAGG 3′, the reverse
primer above, and the high-fidelity polymerase Easy-A Hi-Fi PCR cloning en-
zyme (Stratagene, La Jolla, Calif.) (underlined regions are gene-specific se-
quencies). NsiI or SacI sites were introduced for the purpose of cloning. The
amplified DNA was ligated directly into the corresponding sites of pDXA3C to
generate expression vector pJMS. The construct includes the 5′ ATG of the sglA
gene and is missing the 3′ stop codon. Expression is driven by the actin 15
promoter, and the protein product is myc tagged at the C terminus. Manstein et
al. (26) reported high levels of expression with this vector. The entire sglA frag-
ment was sequenced by using sglA gene-specific primers. Prior to transfor-
mati 0n the plasmid was purified by using a QIAGEN (Valencia, Calif.) Maxi
plasmid preparation kit.

Generation of overexpressor strains. Logarithmically growing D. discoideum
Ax3-ORF2 cells (5 × 10^6 cells/ml) were mixed with 15 µg (10 µl) of vector DNA
and were immediately electroporated as described previously (17). Cells were
brought up to 40 ml in DD broth (26), plated in four 100-mm petri dishes, and
incubated at 22°C for 24 h. Transformants were selected by the addition of 20 µg
of G418/ml at 22°C. Cells were fed every 4 to 5 days with DD broth-G418, and
small colonies of G418-resistant cells began to appear after 10 to 12 days. Control
transformations were Ax3-ORF2 cells transformed with pDXA3C vector. Cells
from 20 individual sglA transformant colonies and from control transformant
colones were transferred to 24-well plates for expansion. These were incubated
for 4 days and then were inoculated both onto 24-well plates containing SM agar
and bacteria in order to examine the phenotype of the cells during growth and
development on the agar and also onto a replica 24-well plate containing DD
broth-G418. Ten single clones, which appeared to be growing more slowly than
the control transformants, were chosen for Western analysis for the presence of
the c-myc-tagged SglA protein. To this end, cells from the corresponding 24-well
plates were plated clonally onto 100-mm-diameter SM agar plates with bacteria
and single clones were isolated.

Western blots. Putative mutant cells were plated on SM agar in association
with K. aerogenes and were allowed to grow to confluence (until the plates were
clear of bacteria). The cells were scraped off the plate, washed twice in SS buffer
(0.6 g of NaCl/liter, 0.75 g of KCl/liter, 0.4 g of CaCl2/liter), pelleted, and kept
frozen at −80°C. Cell pellets were lysed in 1 ml of lysis buffer (50 mM Tris-HCl
[pH 8.0], 5 mM EDTA, 0.5% Triton X-100) including protease inhibitor [1:100
solution of 100× protease inhibitor cocktail, which contained 20 mM 4-(2-
aminoethyl)benzenesulfonyl fluoride, 100 µg of pepstatin A/ml, 10 µg of leu-
petin/ml] on ice. Protein concentration was determined by bieochinonic acid
protein assay (Pierce, Rockford, Ill.). Fifty micrograms of protein was separated
RESULTS

Stable overexpression of S-1-P lyase. The S-1-P lyase null mutant was originally obtained in our random insertional mutagenesis selection for cisplatin resistance. In addition to its increased resistance to the drug, the mutant displayed aberrant development. The aim of this study was to construct mutants that overexpressed the S-1-P lyase to test the hypothesis that altering the levels of S-1-P in the cells would result in increased sensitivity to cisplatin. In addition, we predicted that sglA overexpression would provide additional clues to the role of this enzyme in cell function.

Three mutant strains that overexpress a myc-tagged fusion protein of the S-1-P lyase were isolated. These strains produced the expected 58-kDa fusion protein to different levels of protein. Vector1 and Vector2 are vector control transformants. Ax3-ORF+ is the untransformed parental cells. MW, molecular size standards. (B) Proteins were transferred to nitrocellulose membranes and were probed with anti-c-myc antibodies. (C) The exposed film shown in panel B was scanned and quantitated. The image shows relative units of expression, using sglAOE-3 as a reference (1).
the samples from all three overexpressing strains. The variations in SglA-myc expression are stable and are presumably due to differences in copy number.

**Overexpression of S-1-P lyase results in altered growth regulation.** The S-1-P lyase-overexpressing strains have slower growth rates than the parent strain. This is reflected both in the size of the plaques for bacterially grown cells and in a slower growth rate in liquid. Figure 3A and B depicts the change in plaque size over 3 days for each strain and shows that each overexpressing strain has a different growth rate. The decrease in plaque size reflects the level of overexpression of the SglA protein. The inset in Fig. 3B magnifies the differences at day 3, when the plaques were very small.

Figure 3C shows the growth rate of the strains when growing axenically in HL5 medium. The sglAOE strains also grow more slowly in liquid medium, although the difference becomes less pronounced at densities over $5 \times 10^6$ cell/ml. This growth behavior was observed in three consecutive passages. Again, the degree of growth inhibition is parallel to the level of SglA overexpression, although the differences are not as pronounced as when the cells are growing on agar. Importantly, the cell density at which the cells enter stationary phase is altered in the overexpressing strains, with the higher levels of overexpression causing a lower stationary phase density. SglA overexpression also results in a delayed decrease in cell number in late stationary phase. The change in growth phenotype is accompanied by an increasing number of multinucleate cells, as is shown in Fig. 3D.

**S-1-P lyase overexpression increases sensitivity to the chemotherapeutic agents cisplatin and carboplatin but not to other drugs.** The original sglA mutant had increased resistance to cisplatin, and we hypothesized that the lack of S-1-P lyase resulted in the buildup of S-1-P, a lipid associated with growth promotion and inhibition of cell death. Based on this hypothe-
esis, it is predicted that cells overexpressing S-1-P lyase should be more sensitive to cisplatin. Cells were treated with three concentrations of cisplatin for 4 h, serially diluted, and plated for viability on SM agar in 24-well plates with K. aerogenes as the food source. Viability is expressed as the percentage of surviving cells relative to an untreated culture. slgAΔ is the SlgA null strain. Because the slgAΔ and slgAOE strains had different parents, we established that the Ax3-ORF and Ax4 strains had identical sensitivities to cisplatin (data not shown).

Cross-resistance to drugs is an important aspect of chemotherapy and can considerably limit the options for treatment. Thus, it was determined whether the slgAOE and slgAΔ mutants have increased sensitivity or resistance to other drugs in addition to cisplatin. To this end we tested the response of the two mutants and the Ax4 parent in parallel to a 24-h treatment with cisplatin, carboplatin (a cisplatin analog widely used in chemotherapy), and three other non-platinum drugs (Fig. 5). The data show that the same altered sensitivity to cisplatin was observed in the case of carboplatin. Carboplatin is less toxic to the Ax4 cells (65% survival at 300 μM), but slgAOE-1 is more sensitive to carboplatin and slgAΔ is more resistant to the drug, similar to the results obtained with cisplatin. In contrast, neither mutant showed altered sensitivity or resistance to doxorubicin, 5-FU, or etoposide. Therefore, altering SlgA expression did not affect the cells’ responses to these drugs.

Inhibition of the sphingosine kinase synergistically increases sensitivity to cisplatin. Deleting or overexpressing the slgA gene resulted in increased resistance or increased sensitivity to cisplatin, respectively. These results suggested that inhibiting sphingosine kinase should mimic the slgAOE phenotype and would result in increased sensitivity to cisplatin. To this end we tested the effect of the sphingosine kinase inhibitor DMS on cells of the slgAOE-1, slgAΔ, and parental strains, and the results are shown in Fig. 6.

(i) Parental wild-type cells. High levels of DMS are toxic to D. discoideum cells. Treatment with concentrations of 20 and 50 μM resulted in complete cell death (data not shown), underscoring the importance of sphingosine kinase for normal cell growth. To test the effect of DMS on cisplatin sensitivity, lower doses were chosen, which resulted in a lower level of cell death. Figure 6A and B shows that DMS kills the parental wild-type cells in a dose- and time-dependent manner. Concentrations of 2.5, 5, and 10 μM DMS result in 97, 89, and 28% survival after 5 h and 113, 74, and 11% after 24 h. Treatment of parallel cultures with 150 μM cisplatin alone reduced cell viability to 74% percent after 5 h and 36% after 24 h. Cotreatment of cells with both DMS and cisplatin resulted in a synergistic increase in drug sensitivity. For example, at 5 μM DMS...
FIG. 6. Pretreatment with DMS increased the cell response to cisplatin. Growing cultures of sglAOE-1, sglAΔ, and the parent strain at 10⁶ in HL5 medium were treated with 0, 2.5, 5, and 10 μM DMS for 1 h prior to adding 150 μM cisplatin. The cultures were assayed for viability at 5 and 24 h as described in the legend to Fig. 4. (A and B) parent strain; (C and D) sglAOE-1; (E and F) sglAΔ. Closed circles, 150 μM cisplatin with increasing concentrations of DMS. Open circles, increasing concentrations of DMS alone. (G) Photograph of the plaques of the viable cells at day 3 after plating. Note that increasing concentrations of DMS result in smaller plaques.
survival is reduced 2.8-fold after 5 h and 8.9-fold after 24 h over what would be predicted if the two drugs were working independently. Clearly, inhibiting sphingosine kinase increases sensitivity to cisplatin in the parental cells, as was predicted.

(ii) SglA overexpressor cells. The effect of DMS on the sglAOE-1 cells is shown in Fig. 6C and D. Again, the sglAOE-1 cells were killed by DMS in a dose- and time-dependent manner. As shown above, these cells are initially more sensitive to cisplatin. In contrast to the results with the parental cells, there is no synergistic effect of cotreatment, and the killing with both DMS and cisplatin was not greater than the expected combined killing of the two drugs alone. This is presumably because the level of S-1-P in these cells is already very low due to the increase in the S-1-P lyase enzyme, such that inhibition of the kinase has no additional effect.

(iii) SglAΔ cells. SglAΔ mutant cells are more resistant to cisplatin than the parent cells, and 5 h of treatment with 150 μM cisplatin resulted in virtually no cell killing. Similar to the results described for the parent cells, cotreatment with DMS and cisplatin resulted in a synergistic reduction in viability of 6.5-fold at 5 h and 4.2-fold at 24 h when treated with 5 μM DMS.

Cells treated with DMS produced small plaques, and the size of the plaques decreased with increasing DMS concentrations (Fig. 6G). This is in agreement with the smaller plaque size that was seen with the sglAOE strains and is consistent with the idea that reducing the level of S-1-P results in a decrease in growth rate.

Exogenous S-1-P increases resistance to cisplatin. Based on the results with the sglAOE and null mutants, it was predicted that adding S-1-P to cells would increase resistance to cisplatin—essentially mimicking the sglAΔ mutant. It was previously shown that the developmental timing phenotype of the sglAΔ mutant could be mimicked by adding exogenous S-1-P to wild-type cells (19). The results showed that S-1-P does indeed make parental and sglAOE-1 cells more resistant to cisplatin, as was predicted. Figure 7A and B depicts the results with the parent strain. At 5 h with 150 μM cisplatin there was little killing, and therefore adding S-1-P had no obvious effect. However, at 24 h there was approximately 50% killing with cisplatin alone, and S-1-P at even the lowest dose of 2 μM reversed this to almost the untreated level, i.e., made the cells more resistant. In the case of the initially more-cisplatin-sensitive sglAOE-1 mutant (Fig. 7C and D), S-1-P also increased resistance. The sensitivity to cisplatin was never fully reversed in the sglAOE-1 mutant. This is presumably because of the high levels of S-1-P lyase.

8-Br-cAMP increases resistance to cisplatin. cAMP has been reported to be an activator of sphingosine kinase (25), and this suggested that increasing levels of cAMP could increase resistance to cisplatin in a fashion similar to that of adding S-1-P to the cells. Therefore, the membrane-permeable analog 8-Br-cAMP was used to increase intracellular concentrations of cAMP. 8-Br-cAMP has been shown to be effective at mimicking the effects of cAMP in D. discoideum development, including the late stages of spore development, where 20 mM levels were used for maximum effect (15). The results depicted in Fig. 8 show that 8-Br-cAMP does indeed make both parental and sglAOE-1 cells more resistant to cisplatin. We initially found that high levels of 8-Br-cAMP (10 to 20 mM) were toxic to mitotically dividing cells and therefore chose to test lower concentrations. The effect of 8-Br-cAMP on the
have also identified a cell death via controlling the levels of ceramide, sphingosine, and sphingosine 1-phosphate homolog. Similar mutational strategies with yeast (20). These genes included those encoding sphingosine-1-phosphate kinase, S-1-P phosphatase, and S-1-P lyase enzymes. The resistance of the S-1-P null mutant suggested that this was due to an increase in S-1-P. Thus, we predicted that either increasing the level of the SgIA protein or reducing the activity of the sphingosine kinases would make cells more sensitive to cisplatin. Indeed, overexpression of the S-1-P lyase as well as inhibition of the sphingosine kinases has validated these predictions and strongly suggests that it is the level of S-1-P that ultimately modulates drug sensitivity. The isolation of three stable sglAOE strains that produce different levels of the SgIA protein allowed us to show that the level of expression parallels the level of increased cisplatin sensitivity. The sglAOE-1 strain with the highest level of SgIA overexpression was up to 10-fold more sensitive to cisplatin. Consistent with this result is a recent report that overexpression of S-1-P lyase in HEK-293 cells resulted in increased stress-induced apoptosis (38).

The sglAOE strains also exhibit an expression-dependent decrease of growth rate and stationary phase density and an increase in the average number of nuclei per cell. The increase in the number of nuclei per cell may be related to a decrease in the rate of cytokinesis, which would account for the slower growth rate. Whether the decrease in growth rate directly relates to an increase in drug sensitivity remains to be determined. A similar decrease in cell growth rate has been observed in HEK-293 cells overexpressing the S-1-P lyase gene (38), and yeast lacking the sphingosine kinase gene are delayed from entering S phase (12). The level of protein in the three sglAOE strains that was detected by Western blotting is not precisely linear with the increase in drug sensitivity or growth rate, but it is possible that the signaling systems affected become saturated so that additional expression has no effect.

Treating the cells with the sphingosine kinase inhibitor DMS essentially mimicked the phenotype of the sglAOE strains and showed that modulating the activity of the sphingosine kinases has the predicted effect of also making cells more sensitive to cisplatin. Experiments are in progress with sphingosine kinase null mutants to further test this idea.

The data on the direct addition of S-1-P further strengthens the idea that the pathway of S-1-P synthesis and degradation is intimately involved in modulating the cellular response to cisplatin. Exogenous addition of S-1-P renders the cells more resistant to cisplatin in a manner similar to that of the increase in resistance we observed in the sglAΔ mutant. It was previously shown that exogenous addition of S-1-P to developing Dictyostelium cells can mimic the aberrant developmental phenotype of the sglAΔ mutant (19). S-1-P most likely functions intracellularly after uptake by pinocytosis, because there are no obvious homologs of the G-protein-coupled EDG receptors (SIP receptors) in D. discoideum. This also supports the idea that the changes in cisplatin sensitivity observed in the sglAΔ and sglAOE mutants are due to S-1-P acting as an intracellular second messenger, a function demonstrated in mammalian cells by overexpression of the sphingosine kinase gene in mouse cells lacking the receptors (32).

cAMP has been reported to activate sphingosine kinase (25), and the addition of 8-Br-cAMP to cells had the predicted effect of increasing resistance to cisplatin. It is important to note that...
in a previous paper it was shown that deletion of the \textit{regA} cAMP phosphodiesterase resulted in increased cisplatin resistance (20), and this is in agreement with the discovery that the yeast PDE2 null cells are cisplatin resistant. Thus, cAMP may also be signaling through protein kinase A to modulate cisplatin resistance.

All the previous data show that increasing the S-1-P lyase or decreasing the sphingosine kinase results in altered sensitivity to cisplatin. Consistent with this, sphingosine kinase mRNA has been shown to be overexpressed in a variety of solid tumors (6). This suggests that cotreatment with cisplatin and compounds that lower the levels of S-1-P in cells could result in increased antitumor activity of cisplatin. Cisplatin is generally used in therapy at the maximum allowable dose, and therefore its dosage cannot be increased to treat resistant tumors. Thus, DMS or other sphingosine kinase inhibitors could be useful in combination with cisplatin to increase its efficacy in antitumor therapy—either with tumors such as lymphomas, malignant melanoma, and prostate cancer that do not respond to treatment or in cases such as ovarian tumors that initially respond and then become more resistant (33). Additional sphingosine kinase inhibitors have been reported recently (6), and one inhibitor, phenoxodiol, has been shown to promote apoptosis in ovarian cancer cells (13). Other enzymes of the pathway of sphingosine metabolism conceivably could also be targeted for the purpose of increasing cisplatin sensitivity.

Cross-resistance to drugs is an important aspect of chemotherapy, and it can limit the options for treatment. The specificity of this pathway for the sensitivity to cisplatin is intriguing. The \textit{sglAOE} strains also showed increased sensitivity for the related platinum drug carboplatin but, interestingly, did not show increased sensitivity for other drugs that we tested. Carboplatin is a closely related derivative of cisplatin, which undergoes aquation and has the same platination nucleotide specificity as cisplatin (37). Therefore, it was expected that changing sensitivity to cisplatin would change sensitivity to carboplatin as well, although its toxicity level to wild-type cells is less than that of cisplatin. Indeed, the \textit{sglAOE} cells are more sensitive to carboplatin and the \textit{sglA} cells are more resistant. It will be interesting to see if this relationship holds for the many cisplatin analogs, which have a wide range of toxicities and structures (31). In contrast to the platinum drugs, the \textit{sglAOE} and \textit{sglA} cells did not show altered sensitivity to doxorubicin (DNA intercalator and topoisomerase II inhibitor [2, 5]), 5-FU (inhibits thymidylate synthase and incorporation of fluorodeoxyuridine triphosphates into DNA [22]), and eto-
poside (topoisomerase II inhibitor [35]). Thus, although all three drugs affect DNA metabolism and structure, the resulting cell death does not appear to be modulated by the level of S-1-P lyase. Consistent with this, it was previously shown that the sgd4A mutant did not show significant cross-resistance to UV light, the alkylating agent methylethane sulfonate, or H2O2 (20).

Two major issues arise from these studies: (i) how do sphingolipids affect the cellular response to cisplatin, and (ii) how can these interactions account for the specificity for cisplatin. These questions are particularly important for translating the results from a model system, like D. discoideum, to human tumor cells. Figure 9 presents a model that aims to address these questions and makes predictions for further experiments.

Cisplatin exerts its cytotoxic effect by causing damage to DNA. The DNA damage is recognized by a host of enzymes—some that will target the lesions for repair and others that will target the cell to die. Damaged DNA-dependent protein kinases activate a cascade of signaling that ultimately activates the mitogen-activated protein (MAP) kinase family of proteins, including the MAP kinase ERK family as well as the stress-activated protein kinases of the JNK and p38 families. It has been suggested that the relative balance between p38-JNK and ERK determines the response to cisplatin (40).

The lipids ceramide and S-1-P have also been shown to regulate these MAP kinases. Ceramide activates JNK and p38 and inhibits ERK, while S-1-P activates ERK and downregulates the stress activated enzymes JNK and p38, such that the balance between the MAP kinases reflects the lipids rheostat (4). Thus, in a cell treated with a lethal dose of cisplatin the balance between ceramide and S-1-P usually favors the activation of the p38-JNK enzymes and results in cell death. Based on this model, interfering with the balance of these lipids either by overexpression of the S-1-P lyase or by inhibiting the sphingosine kinase (both of which should result in reduced levels of S-1-P) would increase cell death. In contrast, deleting the S-1-P lyase, activating sphingosine kinase, or adding exogenous S-1-P should result in the downregulation of the JNK- and p38-related proteins and in the upregulation of ERK and should result in decreased sensitivity to the drug (34).

The specificity of the sphingolipid pathway for regulating sensitivity to cisplatin can be attributed to several elements of this model. (i) The cytotoxic effect of cisplatin, but not doxorubicin or taxol, is mediated primarily through p38 (23). (ii) Protein kinase C, phosphatidylinositol 3-kinase, and Akt-PKB are all upregulated by S-1-P (4) and have been linked to cisplatin resistance (11, 40, 46). (iii) Glutathione S-transferase is upregulated by ERK (44). Glutathione S-transferase, in turn, acts as an inhibitor of JNK in addition to interacting with glutathione to directly inactivate cisplatin (10, 14).

Overall, the hypothesis emerging from these studies is that even though the sphingolipid pathway is involved in the response to a number of stresses, the sensitivity of cells to different chemotherapeutic agents may be independently controlled. It is possible that some of these behaviors will end up being cell type and/or drug specific, but together these findings are of considerable significance for the planning of therapeutic strategies when a particular drug is found to be ineffective. We are presently translating the findings of this study to mammalian cells.

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