A Bacterial Protein Enhances the Release and Efficacy of Liposomal Cancer Drugs

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gene expression can be rescued separately by distinct spatial expression patterns of *Bmal1*. Genome-wide profiling experiments suggest that ~10% of the transcriptome is under circadian regulation; however, the majority of these cycling transcripts are tissue-specific (*18*–*22*). Our results are consistent with this tissue-specific diversity of circadian expression and further suggest that core circadian clock components may play distinct roles in different tissues, perhaps in addition to their function in regulating circadian rhythms. The restoration of circadian activity rhythms in brain-rescued *Bmal1*$^{-/-}$ mice is consistent with previous SCN transplant studies in rodents (*23*, *24*). However, the transgenic approach used here has the advantages of preserving the anatomical integrity of the brain as well as allowing the conditional manipulation of the rescue via Dox treatment. The use of tissue-specific and conditional regulation of circadian clock gene expression should be a valuable method for understanding the molecular-, cellular-, and systems-level regulation of circadian rhythms in mammals.

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Supporting Online Material

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A Bacterial Protein Enhances the Release and Efficacy of Liposomal Cancer Drugs

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Clostridium novyi-NT is an anaerobic bacterium that can infect hypoxic regions within experimental tumors. Because *C. novyi-NT* lyses red blood cells, we hypothesized that its membrane-disrupting properties could be exploited to enhance the release of liposome-encapsulated drugs within tumors. Here, we show that treatment of mice bearing large, established tumors with *C. novyi-NT* plus a single dose of liposomal doxorubicin often led to eradication of the tumors. The bacterial factor responsible for the enhanced drug release was identified as a previously unrecognized protein termed liposomase. This protein could potentially be incorporated into diverse experimental approaches for the specific delivery of chemotherapeutic agents to tumors.

There is no dearth of drugs that can kill cancer cells. The challenge is killing the cancer cells selectively while sparing the normal cells. Three basic strategies are currently used to achieve this specificity. The first (selective toxicity) uses drugs that have more potent growth-inhibitory effects on tumor cells than on normal cells (*1*, *2*). This strategy underlies the success of conventional chemotherapeutic agents as well as those of newer targeted therapies such as imatinib (Gleevec). The second strategy (delivery) uses agents such as antibodies that specifically react with molecules that are predominantly expressed in tumor cells (*3*, *4*). The third strategy (angiogenic) exploits abnormal aspects of the tumor vasculature with agents such as bevacizumab (Avastin) (*5*, *6*) or drugs incorporated into liposomes (*7*–*9*). Liposomes are relatively large particles that can penetrate through the fenestrated endothelium present in tumors and a limited number of other organs (*8*, *9*). Once they gain access to tumors, they persist and eventually release their contents and raise local drug concentrations through the enhanced permeabilization and retention effect (*10*). Although each of these strategies has merit, the specificity achieved with any one of them is imperfect, limiting the amount of drug that can be safely administered without causing systemic toxicity.

Here, we describe our efforts to combine all three strategies. We investigated *C. novyi-NT*, an attenuated strain of the obligate anaerobe *C. novyi*. Similar to other bacteriolytic therapies, *C. novyi-NT* can selectively infect and partially destroy experimental cancers because of the hypoxic nature of the tumor environment (*11*, *12*). *C. novyi-NT* is also hemolytic (lyses erythrocytes). Because enzymes that rupture erythrocytes can disrupt lipid bilayers (*13*), we hypothesized that the bacterium's hemolytic properties could be exploited to enhance the release of liposome-encapsulated drugs within tumors. This approach would theoretically increase specificity by combining the selective tumor toxicity of chemotherapeutic agents, the selective delivery of *C. novyi-NT* to tumors, and the selective uptake of liposomes mediated by the abnormal tumor vasculature.

To test this hypothesis, we first treated syngeneic CT26 colorectal tumors in BALB/c mice. *C. novyi-NT* spores were injected intravenously, and once germination had begun in the tumors (~16 hours after injection), we administered a single intravenous dose of liposomal doxorubicin (Doxil). Doxil is a liposomal formulation that encapsulates doxorubicin, a widely used DNA-damaging chemotherapeutic agent. Liposome-encapsulated doxorubicin has been shown to result in improved outcomes compared with unencapsulated doxorubicin (*14*). As previously documented (*15*, *16*), treatment with *C. novyi-NT* spores alone resulted in germination and necrosis within the centrally hypoxic region of tumors but left a well-oxygenated viable rim that eventually regrew (Fig. 1A). Neither doxorubicin nor Doxil alone resulted in prolonged therapeutic effects in these mice. The combination of Doxil and *C. novyi-NT* spores, however, resulted in complete regression of tumors in 100% of mice (Fig. 1A), and 65% of the mice were still alive at 90 days (Fig. 1B).

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Notably, 100% of the mice treated with *C. novyi-NT* and free doxorubicin at the same dose died within 2 weeks, emphasizing the crucial role of liposomal encapsulation in reducing systemic toxicity (*14*). Similar antitumor effects were observed in experiments with immunodeficient mice bearing human colorectal cancer xenografts (Fig. 1, C and D). Heat-inactivated *C. novyi-NT* spores did not lead to enhanced tumor regression when injected in combination with Doxil (fig. S1).

We next compared the distribution of doxorubicin in mice treated as in Fig. 1. Administration of *C. novyi-NT* spores plus Doxil resulted in tumor drug exposure that was approximately six times the exposure achieved with Doxil alone, without increasing drug concentrations in normal tissues (Fig. 2). This effect was specific to *C. novyi-NT* and not the result of inflammation per se, given that intratumoral injection of the potent inflammatory agent lipopolysaccharide (LPS) before the administration of Doxil did not affect the intratumoral concentration of doxorubicin (fig. S2A). *C. novyi-NT* spores did not enhance the accumulation of unencapsulated doxorubicin, excluding the possibility that the increase in accumulation of Doxil was simply due to changes in blood flow associated with infection (fig. S2B). After treatment with Doxil and *C. novyi-NT*, doxorubicin was released from liposomes and was bound to tumor cell nuclei, as revealed by fluorescence microscopy (fig. S3).

We next attempted to identify the mechanism through which *C. novyi-NT* enhances intratumoral release of liposome-encapsulated doxorubicin. Culture medium conditioned by the growth of *C. novyi-NT* contained substantial liposome-disrupting activity and the concentration of this factor was maximal in late log-phase (fig. S4, A and B). We anticipated that this secreted factor would be a phospholipase, because these enzymes are known to disrupt the lipid bilayers of liposomes as well as those of erythrocytes (*13*). However, hemolytic activity by phospholipases was not required for the liposome-disrupting activity (fig. S5).

To identify the liposome-disrupting factor, we fractionated the growth medium from late log-phase cultures by means of a combination of ammonium sulfate precipitation, ion exchange chromatography, and gel filtration. A single, major peak of liposome-disrupting activity was observed (Fig. 3, A and B) and SDS-polyacrylamide gel electrophoresis revealed a predominant protein of approximately 45 kD in the active fractions (Fig. 3C). This protein was purified, digested by trypsin, and analyzed by liquid chromatography–tandem mass spectrometry. Using the *C. novyi-NT* genome as reference (*17*), we identified the polypeptide as a putative neutral lipase encoded by the NT01CX2047 gene (fig. S6). This gene was not highly homologous to its closest counterpart in other bacteria (50% amino acid identity to a *C. tetani* lipase).

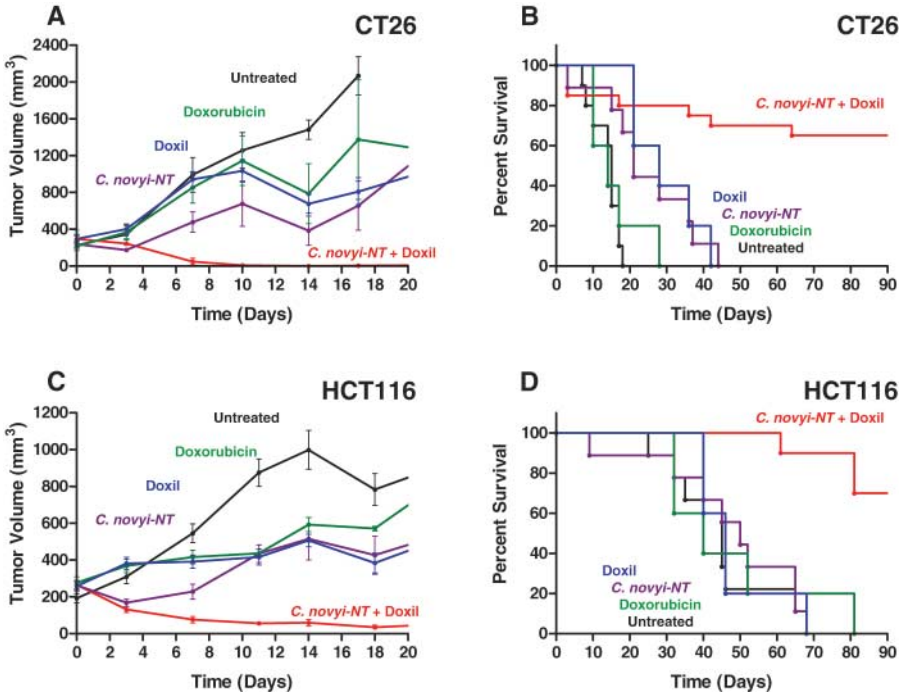

Fig. 1. *C. novyi-NT* treatment enhances the antitumor activity of Doxil in mice. Mice bearing the indicated tumors were treated with various combinations of the indicated agents, and the effects on tumor volume (**A** and **C**) and survival rates (**B** and **D**) were observed. Treatment with doxorubicin plus *C. novyi-NT* spores was toxic and resulted in deaths of all animals within two weeks (not shown). Means and standard errors of data collected from at least five mice per group are illustrated. The differences between *C. novyi-NT* plus Doxil and all other groups were significant ($P \leq 0.0006$, log-rank test) in the survival curves.

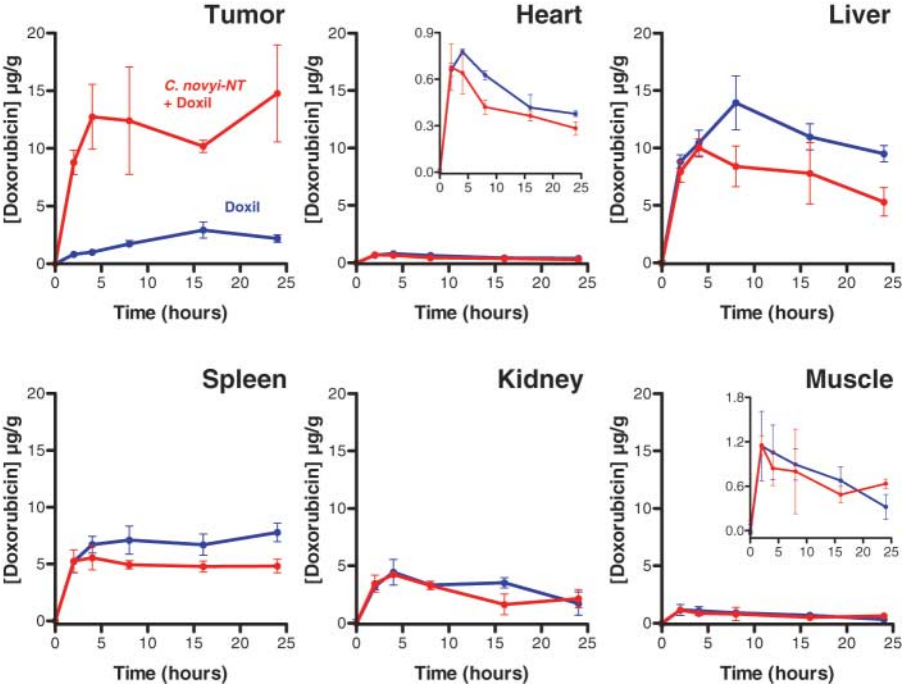

Fig. 2. Pharmacokinetic distribution of doxorubicin after treatment with *C. novyi-NT*. Doxil was administered to athymic nude mice bearing HCT116 tumors that were ~300 mm^3 in size. Another group of mice was intravenously injected with *C. novyi-NT* spores 16 hours before Doxil treatment. Mice were sacrificed at the indicated time points after Doxil administration and doxorubicin was extracted from tissues and measured by fluorometry. Means and standard deviations from three mice per time point are shown. Insets in heart and muscle panels were rescaled to show differences.

The identification of the liposome-disrupting factor as the product of NT01CX2047 was consistent with the evidence that this gene was preferentially expressed in late log-phase and was highly expressed in tumors after infection with *C. novyi-NT* (fig. S4C). Additionally, based on its sequence, the product of NT01CX2047 was predicted to be secreted (fig. S6).

To examine the properties of the protein encoded by NT01CX2047, we cloned its open reading frame into an inducible expression vector which was then introduced into a genetically modified *Escherichia coli* strain that permitted expression of *C. novyi-NT* genes (which have a codon usage very different than that of *E. coli*). After induction of protein expression by isopropyl-β-D-thiogalactopyranoside, the transformed *E. coli* cells grew poorly, presumably because the gene product was toxic. Lysates from these cells were tested for lipase activity as measured by hydrolysis of 1,2-dioleoyl-3-pyrenedecanoyl-*rac*-glycerol. The NT01CX2047-expressing clones exhibited lipase activity, whereas clones cured of the vector (*18*) did not (Fig. 4A). As further controls, we generated two mutants: one in which a stop codon was substituted for serine at amino acid 127 (S127X), and another in which this serine was replaced by glycine (S127G). Ser^{127} is found within the highly conserved GXSXG lipase motif and was predicted to be the essential catalytic serine responsible for lipase activity (*19*). Both the S127G missense mutant and the S127X truncation mutant were devoid of lipase activity (Fig. 4A), although each was expressed at levels comparable to those expressing the wild-type NT01CX2047 polypeptide (fig. S7). Notably, cells expressing the noncatalytic S127G mutant exhibited the same poor growth as those expressing wild-type NT01CX2047, whereas cells expressing the S127X mutant grew as well as cured cells.

To test the liposome-disrupting activity of these engineered *E. coli* lysates, we incubated them with Doxil. Lysates containing wild-type NT01CX2047 showed potent activity in this assay, whereas lysates from cured cells showed no activity (Fig. 4B). As expected, the S127X truncation mutant had no detectable liposome-disrupting activity, but surprisingly, the S127G mutant, which was devoid of lipase activity, retained substantial liposome-disrupting activity (Fig. 4B). To determine whether this activity was a general feature of lipases or specific to the NT01CX2047 lipase of *C. novyi-NT*, we assayed nine commercially available enzymes with well-defined lipase activity; none had significant liposome-disrupting activity (Fig. 4C).

The absence of a requirement for enzymatic activity implied that the liposome disruption mediated by the NT01CX2047 gene product might be due to a physical process. To investigate this possibility, liposomes containing 1,6-diphenylhexatriene or Laurdan as membrane-sensitive probes were used to investigate the effects of NT01CX2047 on lipid order and membrane polarity, respectively. Lipid order measured by fluorescence polarization significantly decreased with increasing concentrations of NT01CX2047 (fig. S8A). The perturbation of membrane structure by the protein was similar to that mediated by alcohols, although the protein was orders of magnitude more potent on a molar basis (fig. S8A). Membrane polarity measured by fluorometry also increased in the presence of NT01CX2047, indicating greater access of polar molecules to the lipid bilayer matrix (fig. S8B).

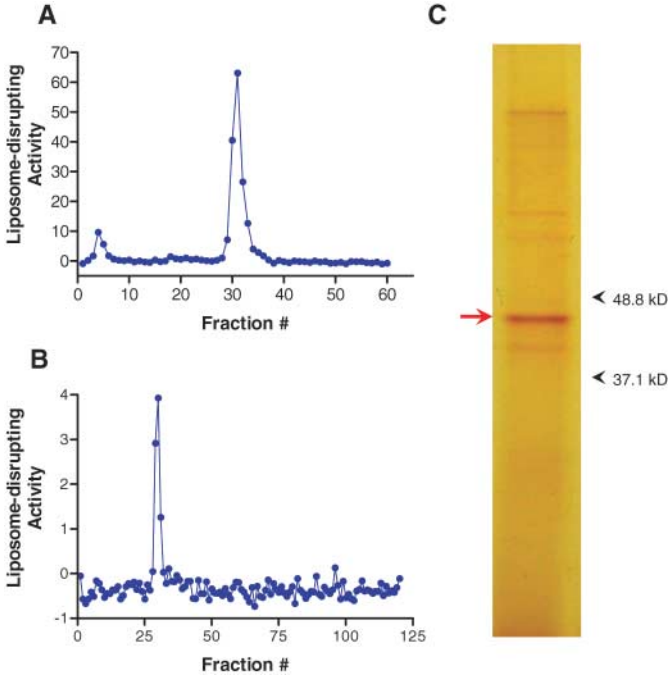

Fig. 3. Biochemical purification and identification of liposome-disrupting activity from *C. novyi-NT*. (**A**) *C. novyi-NT* was grown until late log-phase and the medium cleared of cells by centrifugation. After precipitation with 40% saturated ammonium sulfate, ion exchange chromatography was performed and fractions evaluated for liposome-disrupting activity. (**B**) The peak fractions (30 to 31) from (A) were pooled and further fractionated by gel filtration chromatography. (**C**) The peak fractions (29 to 30) from (B) were subjected to SDS-polyacrylamide gel electrophoresis. Silver-staining of the gel revealed a predominant band migrating between protein standards with masses 48.8 and 37.1 kD.

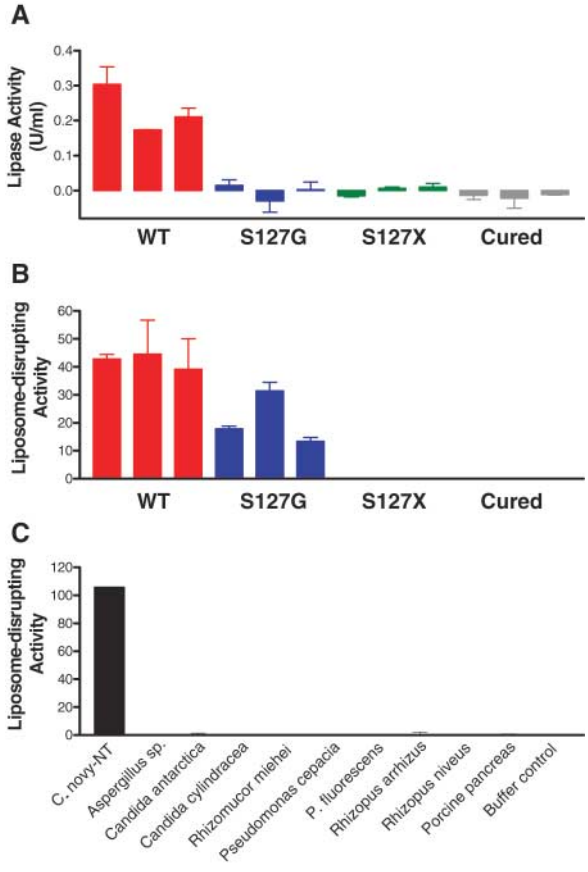

Fig. 4. Functional analysis of the *C. novyi-NT* lipase. Plasmids carrying the wild-type or mutant forms of the NT01CX2047 gene were introduced into *E. coli*. "Cured" bacteria were grown in the absence of antibiotics until the plasmid was lost. Cellular lysates from three independent clones of each strain were assayed for lipase activity (**A**) and liposome-disrupting activity (**B**). (**C**) Liposome-disrupting activities of lipases purified from various organisms were assessed with the use of Doxil. Means and standard deviations of data from at least two independent experiments are shown.

These results suggest that the liposome-disrupting activity of the protein encoded by NT01CX2047 (henceforth termed liposomase) is due to interaction of its lipid-binding domain with the liposomal membrane and the consequent alteration of bilayer structure. This explains why a lipase, which has little phospholipase activity, can disrupt liposomes, which contain phospholipids but no triacylglycerides.

In principle, the approach described here should be applicable to any chemotherapeutic drug that can be encapsulated in a liposome. Indeed, when we repeated the preclinical efficacy experiments with liposomes carrying CPT-11 (irinotecan), a topoisomerase inhibitor widely used in cancer therapy, we obtained results similar to those in the Doxil experiments (fig. S9). Both CT26 mouse tumors and HCT116 human xenografts were relatively resistant to CPT-11 when the drug was administered alone in unencapsulated or liposome-encapsulated form. However, when liposomal CPT-11 was delivered in combination with *C. novyi-NT* spores, all tumors regressed and more than 60% of the mice survived for at least 3 months (fig. S9). Notably, the combination therapy was effective against small (136 mm^3 in volume) as well as large tumors. In previous studies, small tumors were resistant to bacteriolytic therapies because they had relatively small regions of necrosis (*15*). Importantly, mice treated with *C. novyi-NT* and either Doxil or CPT-11 tolerated the treatments well and did not generally suffer toxicities or weight loss (fig. S10).

We have not excluded the possibility that other secreted factors may contribute to the liposome-disrupting activity. However, the data reported here suggest that liposomase substantially contributes to the therapeutic effects observed in vivo. The identification and cloning of liposomase opens the door to therapeutic strategies in addition to those based on bacteriolysis. For example, liposomase could be attached to antibodies or encoded within vectors used for gene therapy (*3*, *4*). Because virtually any therapeutic agent can be packaged in liposomes and can thereby act as a "prodrug," liposomase offers a number of possibilities for the specific delivery of drugs to tumors.

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Predictive Codes for Forthcoming Perception in the Frontal Cortex

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Incoming sensory information is often ambiguous, and the brain has to make decisions during perception. "Predictive coding" proposes that the brain resolves perceptual ambiguity by anticipating the forthcoming sensory environment, generating a template against which to match observed sensory evidence. We observed a neural representation of predicted perception in the medial frontal cortex, while human subjects decided whether visual objects were faces or not. Moreover, perceptual decisions about faces were associated with an increase in top-down connectivity from the frontal cortex to face-sensitive visual areas, consistent with the matching of predicted and observed evidence for the presence of faces.

One function of the visual system is to decide what is present in the local environment, resolving potentially ambiguous sensory information into a coherent percept. Models of perceptual decision-making propose that specialized detectors accumulate evidence in favor of a preferred feature or representation, and the output of these detectors is compared in a winner-takes-all fashion at a downstream processing stage (*1*). Accordingly, when subjects are asked to decide whether they perceive stimulus A or stimulus B, cell assemblies in the frontal and parietal cortices track the difference in output of visual neurons collecting evidence in favor of A and B (*2*, *3*).

Prior information may help the brain decide among competing percepts (*4*). According to one view, the brain generates "predictive codes" that dynamically anticipate the forthcoming sensory environment, weighting perceptual alternatives on the basis of this prediction (*5*, *6*). Predictive accounts of decision-making have long been embedded in theories of signal detection, which suggest that subjects compare observed sensory evidence against an internal "template," with a response elicited if the match between the evidence and the template reaches a given criterion (*7*). Moreover, predictive coding offers a parsimonious account for several well-known behavioral phenomena (*8*–*10*) and recent neurophysiological findings (*11*, *12*). The theory requires that decision-making neurons have access to the set of predicted information (here, we call this "perceptual set") against which to match the sensory data. However, little is known about how—or where—perceptual set might be represented in the decision-making architecture of the brain.

To address this question, we capitalized upon recent work in which functional magnetic resonance imaging (fMRI) was used to identify brain regions responsible for collecting evidence about the presence of faces on

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