Abstract

One of the major challenges facing cancer therapy today is achieving specificity. Current efforts to meet this challenge are focused on developing targeted therapeutics specific to the cancer cell. An alternative approach is to selectively deliver cytotoxic agents to the tumor site. With this end in mind, liposomes optimized for physical robustness have been developed and used clinically as drug delivery vehicles. Paradoxically, the effectiveness of these liposomes is hampered by the suboptimal release of bioavailable drug. This article will highlight the recent advance in using a novel lipase secreted by the tumor-colonizing anaerobic bacterium *Clostridium novyi*-NT to induce the targeted release of liposomal payloads within tumors. [Cancer Res 2007;67(20):9605–8]

Liposomal Bullets

The goal of cancer therapy is to kill cancer cells and spare healthy ones. This is easier said than done. Cancer cells are often insensitive to particular therapies. Yet, increasing doses to achieve a therapeutic effect is not an option if toxicity to healthy tissues becomes limiting. One early attempt to reduce systemic toxicity used liposomes to encapsulate cytotoxic drugs (1). However, these liposomes were rapidly eliminated by the mononuclear phagocyte system. This limitation was abrogated by the use of a unique liposomal formulation combining PEGylated phospholipids, high cholesterol content (40–50% mol/mol), and hydrogenated phospholipids (2). These liposomes, called sterically stabilized liposomes (SSL), had several characteristics. The abundant cholesterol and use of hydrogenated phospholipids resulted in a high bilayer phase-transition temperature and general physical rigidity, thus preserving the physical integrity of these liposomes as they traversed the circulation (3, 4). Additionally, PEGylation markedly reduced the uptake of these liposomes by the mononuclear phagocyte system, resulting in an improved circulation half-life (5). This extended circulation time opened a new possibility—exploiting the fenestrations in tumor vessels resulting from aberrant angiogenesis. These pores, varying between 100 and 780 nm in diameter, are significantly larger than the gaps found in normal endothelium, which are typically <6 nm wide (6). Liposomes could thus be sized large enough to be excluded from normal tissues, yet small enough to passively infiltrate tumor endothelium and deliver their cargo. This phenomenon, called the enhanced permeability and retention effect, was found to be of therapeutic benefit in multiple tumor models (7). Thus, SSLs exhibited low toxicity and consequently, the ability to be administered at higher drug doses. Doxil, a clinically used SSL formulation of doxorubicin with a greatly improved cardiotoxicity profile, is the prototype example. More recently, tumor-targeting molecules (e.g., antibodies, integrins, folate) have been conjugated to these liposomes to improve their retention within tumors expressing their cognate molecular targets (7).

Paradoxically, the physical robustness of SSLs, a desirable trait for keeping the drug compartmentalized and sequestered, also proved to be a double-edged sword. SSLs retained in tumors degraded at a slow rate, leaking their therapeutic cargo over a prolonged period of time, making it difficult to achieve the high concentrations necessary for provoking a therapeutic response (4). This explains why the inherent low toxicity of the SSL approach did not intuitively translate into dramatically improved efficacy, even though greater treatment doses than before were now possible (8). Consequently, a recent direction of liposome research has been to engineer liposomes that would be selectively destabilized within tumors.

One approach to effect this destabilization is to dope the liposomal bilayer with pH-sensitive phospholipids and ligands that bind internalizing receptors highly expressed on tumor cells. Binding of these targeted liposomes to their cognate receptors initiates internalization of the liposomes by receptor-mediated endocytosis. Subsequently, the pH-sensitive liposomal components become destabilized within acidified endosomal compartments augmenting the release of encapsulated drugs (9). These liposomal formulations (even with the inclusion of PEGylated lipids) have significantly shorter circulation times than SSLs, but this is offset by the potential benefit of more liposomal drug being released and thus made bioavailable. A second example exploits phospholipase A2 (PLA2), a lipid-metabolizing enzyme elevated in tumor tissue (10). However, PLA2 is not expressed exclusively in tumor tissue. Another limitation of this approach is that PLA2 is unable to degrade SSLs because of their high (~50%) cholesterol content. Decreasing this cholesterol level to 20% or less would make SSLs degradable by PLA2, but would also decrease their rigidity and drug retention capability. Alternative approaches depend on an external source of destabilization directed toward the tumor. This destabilization may be caused by physical means, for example, ultrasound, light, or hyperthermia directed from external sources (7). The drawback to these approaches is that prior knowledge of tumor location is required, which makes this method unsuitable for treating disseminated disease.

The above examples collectively define the challenge for liposomal drug design—to achieve targeted and rapid drug release in tumors without compromising the need for low toxicity. A unique opportunity for achieving this goal emerged from recent research on tumor-colonizing anaerobic bacteria.

*Clostridium novyi*-NT, the Scope

The tumor microenvironment is not physiologically homogeneous but harbors hypoxic regions (11, 12). Because these regions
are highly inaccessible to systemic therapy and resistant to radiation, tumor hypoxia is a prognostic indicator of poor treatment outcomes. On the flip side, hypoxia is a defining characteristic of solid tumors that can be exploited for cancer therapy (12). One approach involves the use of anaerobic bacteria that selectively proliferate in hypoxic tumor tissues (13). Anaerobic bacteria have been used as anticancer agents for more than half a century. In 1947, Parker et al. (14) treated mice bearing transplanted sarcomas with Clostridium histolyticum spores. Subsequently, a strain of the anaerobic bacterium Clostridium sporogenes was isolated and tested in animal tumor models (15). Tumor lysis was observed in spore-treated animals. These encouraging results eventually led to clinical trials in which the C. sporogenes spores were used to treat a limited number of patients with different cancer types (16). Although initially promising, these early studies did not show substantial therapeutic benefit and sometimes showed excessive toxicity.

Clostridium novyi-NT (C. novyi-NT) is a Clostridium strain that has shown substantial therapeutic potential in preclinical models. Wild-type C. novyi was first chosen for further investigation from a panel of candidate therapeutic bacterial species because of its ability to invade necrotic/hypoxic tumor tissue after germination (17). However, C. novyi was found too toxic and thus engineered to generate an attenuated strain, C. novyi-NT, in which the lethal z-toxin gene was deleted (17). When administered i.v., C. novyi-NT spores germinate only within tumors and spread rapidly throughout necrotic/hypoxic tumor tissue, causing hemorrhagic necrosis and tumor regression (18, 19). Although effective against cells in hypoxic regions, C. novyi-NT alone is inefficient when encountering the well-perfused and oxygen-rich tumor tissue located in the tumor rim. Consequently, the tumor rims often grow back, leading to relapse. This suggested that an ideal therapeutic regimen should include components that target both well-perfused and hypoxic tumor compartments. Indeed, when combined with chemotherapy, C. novyi-NT treatment could cure a considerable fraction of tumor-bearing animals (17, 20). However, the drug doses required for such efficacy often generated substantial toxicities. Fortuitously, C. novyi-NT possessed a unique device, allowing targeted release of SSL-encapsulated drugs in tumors, thereby increasing efficacy while diminishing the toxicity of chemotherapy delivered in conjunction with C. novyi-NT.

**Liposome, the Trigger**

It is well documented that C. novyi is a hemolytic bacterium. We hypothesized that this membrane-disrupting property could be exploited to enhance the release of liposome-encapsulated drugs within tumors. Consistent with this idea, culture medium conditioned by the growth of C. novyi-NT was able to cause the release of doxorubicin from Doxil (21). When mice bearing large,
established tumors were treated with \textit{C. novyi-NT} plus a single dose of SSL-encapsulated doxorubicin or irinotecan, complete regression of tumors was observed in all mice and more than two thirds of the animals were cured. In comparison, treatment with \textit{C. novyi-NT} or SSL-encapsulated drugs alone did not show sustained therapeutic effects. Notably, all mice treated with \textit{C. novyi-NT} plus free doxorubicin died within 2 weeks, underscoring the importance of liposomal encapsulation for reducing systemic toxicity. The secreted factor responsible for liposomal disruption was purified by chromatographic methods and identified as a new lipase (termed liposomase), not a conventional hemolysin. Interestingly, the secreted liposomase was found to act through physical perturbation of membrane lipid order and not through catalytic degradation of membrane lipids, suggesting a novel mechanism for liposomal drug release. Nine commercially available enzymes with well-defined lipase activities were tested and none exhibited liposome-disrupting activity, demonstrating that this phenomenon was not a general characteristic of lipases.

Phylogenetic analysis of these nine lipases reveals that they are relatively distant to liposomase and this may account for their lack of liposome-disrupting activity (Fig. 1, green box). In comparison, the closest relatives to liposomase as identified by the BLAST algorithm and plotted on the same dendrogram include lipases from \textit{Clostridium tetani} and various thermophilic bacilli (Fig. 1, blue box). These lipases are not commercially available and it would be interesting to see if their similarity to liposomase correlates with liposome-disruption capability.

One of these close relatives of liposomase, \textit{Bacillus steatothermophilus} lipase, recently had its structure elucidated by X-ray crystallography, thus enabling the prediction of a theoretical structure for liposomase by homology modeling (Fig. 1, inset). As is consistent with lipases in general, the liposomase model features a stretch of hydrophobic residues forming a lid that conceals the catalytic site (Fig. 1, inset, catalytic residues in green). In other lipases, this lid plays a key role by adopting an “open-lid” conformation upon contact with a water-lipid phase boundary to allow access of lipids to the catalytic center. One tantalizing possibility is that physical interaction between the open hydrophobic lid of one liposomase molecule and the exposed catalytic area of another open molecule might enable the self-assembly of liposomase molecules into multimeric structures able to disrupt lipid membranes (22). This remains a hypothesis to be tested.

**Implications and Future Directions**

Recent advances in molecular biology have allowed the engineering of tumor-targeting bacterial strains that express heterologous proteins (13, 23, 24). For example, different \textit{Bifidobacterium}, \textit{Clostridium}, and \textit{Salmonella} strains have been engineered with therapeutic genes for prodrug-converting enzymes (e.g., thymidine kinase, cytosine deaminase, nitroreductase), immunomodulatory factors (e.g., interleukin 2, tumor necrosis factor-\alpha), and angiogenesis inhibitors (e.g., endostatin). Liposomase does not replace any of these approaches and could potentially complement and improve their potency. For example, bacteria engineered to express both liposomase and prodrug-converting enzymes would simultaneously enable the specific release and metabolic activation of liposome-encapsulated prodrugs within tumors.

Other implications of this research are worth noting. First, now that liposomase has been identified and cloned, it can be used in a variety of other targeted therapies in addition to those using bacteria. For example, liposomase could be attached to antibodies or ligands that have an affinity for specific tumor markers. Alternatively, the liposomase gene could be incorporated into gene therapy vectors. As most drugs can be packaged within liposomes, these approaches would have general utility. Second, approaches using liposomase may be particularly relevant for delivering toxic biologics, such as cytokines and toxins. SSL encapsulation coupled with targeted liposomase-mediated drug release could minimize the systemic inflammation and toxicity that currently limits their clinical use while achieving therapeutic concentrations at the disease site. Third, the mechanism underlying liposome disruption by liposomase is currently a black box. Understanding this physical phenomenon may allow us to design liposomes or other polymeric carriers that are more susceptible to liposomase, further increasing its therapeutic value. Fourth, this study shows that balancing the desire for drug efficacy against the need to minimize toxicity is not necessarily a zero-sum game if specific drug delivery can be achieved.

Most cancer drugs fail in clinical studies not because they are ineffective in killing cancer cells. Rather, the problem is that one cannot give doses high enough to eradicate the tumor without also killing the patient. In essence, a rifle, not a shotgun, is needed in cancer therapy. The use of liposomase secreted by the tumor-colonizing \textit{C. novyi-NT} as a molecular trigger for targeting the release of liposomal drugs is one way to build this precision weapon. Because this technology is not limited to any one particular tumor type or drug, its potential to “rescue” toxic drugs and improve already successful drugs is therefore very broad.

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