Tumor Cell Killing Enabled by Listeriolysin O-liposome-mediated Delivery of the Protein Toxin Gelonin*

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Gelonin is a type I plant toxin that has potential as an effective anti-tumor agent by virtue of its enzymatic capacity to inactivate ribosomes and arrest protein synthesis, thereby effectively limiting the growth of cancer cells. Being a hydrophilic macromolecule, however, gelonin has limited access to its target subcellular compartment, the cytosol; it is effectively plasma membrane-impermeant and subject to rapid degradation within endosomes and lysosomes upon cellular uptake as it lacks the membrane-translocating capability that is typically provided by a disulfide-linked B polypeptide found in the type II toxins (e.g. ricin). These inherent characteristics generate the need for the development of a specialized cytosolic delivery strategy for gelonin as an effective anti-tumor therapeutic agent. Here we describe means of delivering gelonin into the cytosol of B16 melanoma cells. Gelonin was co-encapsulated inside pH-sensitive liposomes with listeriolysin O, the pore-forming protein that mediates escape of the intracellular pathogen Listeria monocytogenes from the endosome into the cytosol. In vitro experiments, co-encapsulated listeriolysin O enabled liposomal gelonin-mediated B16 cell killing with a gelonin IC50 of ~0.1 nM with an extreme efficiency requiring an incubation time of only 1 h. By contrast, cells treated with equivalent concentrations of unencapsulated gelonin or gelonin encapsulated alone in pH-sensitive liposomes exhibited no detectable cytotoxicity. Moreover, treatment by direct intratumor injection into subcutaneous tumors of B16 melanoma in a mouse model showed that pH-sensitive liposomes containing both listeriolysin O and gelonin were more effective than control formulations in curtailing tumor growth rates.

Delivery of exogenous macromolecules to the cytosol is a fundamentally inefficient process. This difficulty arises from the fact that cells have an obligation to maintaining homeostasis; hence the need for strict control over what is allowed passage, intact, into the cell. Because of their hydrophilicity and large hydrodynamic volumes, macromolecules such as DNA and protein are effectively impermeant to the cell’s plasma membrane. Those that are taken up by cells via, for example, fluid-phase or receptor-mediated endocytosis are ultimately degraded within the endosomal/lysosomal pathway, or in some cases are returned to the extracellular environment (1, 2). The ultimate fate of internalized macromolecules may not be of concern where the site of action is at the cell surface (e.g. insulin). For many biomolecules with therapeutic potential, however, direct interaction with an intracellular target may be a prerequisite for efficacy. This condition is particularly true for many plant-derived toxins that have cytostatic or cytotoxic activities and thus have potential as anti-cancer therapies.

Plant toxins currently used or envisioned in pharmaceutical formulations are predominantly either type I or type II toxins. The type II toxins are composed of two disulfide-linked polypeptides (A and B). The B chain binds to the plasma membrane and facilitates entry of the catalytic A chain into the cytosol where it inhibits protein synthesis by irreversibly depurinating the 28 S rRNA in the 60 S subunit of the host cell’s ribosome (3). Type I toxins lack the B chain and hence the mechanism for efficient entry into the cytosol (3). Gelonin, a 30 kDa type I toxin from the seeds of Gelonium multiflorum, is an ideal candidate for therapeutic delivery due to its minimal toxicity when added extracellularly (4). Numerous strategies have been employed to efficiently deliver gelonin to the cytosol of cancer cells including conjugation to folate, hormones or monoclonal antibodies, and entrapment in liposomes or polymers (5–14). These approaches are predominantly aimed at increasing cell-type-specific binding and uptake and in some cases augmenting delivery into the cytosol. Although uptake can be enhanced, most of the currently available formulations have a tendency to mediate delivery into the endocytic pathway but lack the ability to promote efficient escape of the protein from endosomes into the cytosol.

Our approach is to co-encapsulate gelonin with listeriolysin O (LLO) in pH-sensitive liposomes. This delivery system mimics the intracellular invasion strategy of the bacterial pathogen Listeria monocytogenes, which uses LLO to escape degradation within the endosomal/lysosomal pathway by facilitating entry of the bacterium into the cytosol (15–17). LLO has been used alone as a delivery facilitator in recombinant non-pathogenic strains of bacteria, or in combination with pH-sensitive liposomes to successfully deliver antigenic protein, oligodeoxynucleotides, and plasmid DNA to the cytosol (18–24). Co-encapsulation of the drug along with LLO in liposomes in principle would provide a certain amount of in vivo protection.
against potential side effects of the cargo. Moreover, a liposome-based delivery platform has the advantages of protecting the encapsulated protein from degradation in plasma, high payload per event, potential for modifying the pharmacokinetics and prolonging plasma circulation, and targetability upon incorporation of specific ligands or monoclonal antibodies (25–30). Our results show that LLO is required for efficient delivery of gelonin from pH-sensitive liposomes to the cytosol of B16-F10 murine melanoma cells. Furthermore, this formulation is more liposensitive than either free gelonin or gelonin encapsulated alone in pH-sensitive liposomes, reducing the growth rate of tumors when directly injected into solid tumors of melanoma subcutaneously growing in mice. This is also the first demonstration that the combination of LLO and pH-sensitive liposomes can be used to deliver an active enzyme to the cytosol.

**EXPERIMENTAL PROCEDURES**

**LLO Purification**—Recombinant His-tagged LLO (57 kDa) was purified from an *Escherichia coli* expression system as previously described (30). Briefly, *E. coli* strain BL21 (DE3) pLysS (Promega Corp., Madison, WI) were transformed with a C-terminally His-tagged version of the LLO cDNA (a generous gift from Dr. D. Portnoy, University of California, Berkeley, CA) in the pET29b vector (Novagen, Inc., Madison, WI). Bacteria from a single colony were grown in Luria-Bertani broth for 16 h at 30 °C, then diluted 1:25 and expanded at 30 °C to log phase (A570 = 0.6), at which point expression of LLO was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM for four hours. All steps from this point on were performed at 4 °C unless otherwise noted. Cells were collected by centrifugation and lysed using a French press (Thermo Electron, Rochester, NY). The lysate was centrifuged at 12,000 × g for 40 min in a Beckman J2-21 M high-speed centrifuge (Beckman Coulter, Inc., Fullerton, CA) and the supernatant incubated with nickel-nitrotriacetic acid agarose (Qiagen, Inc., Valencia, CA) for 1 h. The agarose was extensively washed (4 × 200 bed volumes) and the His-tagged LLO eluted with 0.5 M imidazole (Sigma). The eluate was dialyzed extensively against Tris-buffered saline (10 mM Tris-Cl, 140 mM NaCl, pH 8.5, at 4 °C), and the activity of purified LLO was confirmed by assaying for its ability to lyse sheep red blood cells, as detected by a decrease in right-angle scattering of 590 nm light by the cells in a Fluoromax-2 fluorometer (Instruments S.A., Inc., Edison, NJ) (22). The molecular weight and relative purity of the recombinant LLO were confirmed by SDS-PAGE and Coomassie staining, and protein concentration determined by the BCA assay (Pierce).

**Preparation of Liposomes**—All of the liposomes used in this report were prepared following the method previously used and tested for LLO delivery (32, 33, 34). In brief, 5 mol % of PE was replaced with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (PE, Avanti Polar Lipids, Inc.), were prepared in an identical manner to the liposome formulation was typically 150–200 nm (±5 nm S.D.). Assuming an average surface area of individual phospholipid head groups in liposomes of roughly 60 Å², the amount of gelonin encapsulated in purified liposomes is estimated to be ~15–20 gelonin molecules per 200 nm liposome.

**Animals and Cell Viability Assay**—C57BL/6J (B6) mice (The Jackson Laboratory, Bar Harbor, ME), 6-week-old females, were housed and cared for according to the National Institutes of Health Laboratory Animal Medicine guidelines. A B6-derived murine melanoma cell line, B16, was the generous gift of Dr. K. L. Rock (University of Massachusetts, Worcester, MA). For *in vitro* viability assays, B16-F10 cells were plated at 5 × 10⁴ cells per well in a 96-well plate in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated (55 °C for 45 min) fetal bovine serum, 10 units/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES in a humidified 37 °C incubator at 5% CO₂. After a 24-h incubation, cells were pulsed with one of three liposome formulations containing LLO alone, gelonin alone, or both gelonin and LLO, for 1 h in serum-free media. The cells were washed once and the media replaced with phenol red-free complete media. Sodium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) labeling reagent and N-methyl dibenzoazepine methyl sulfide (Sigma) electron coupling reagent were added at final concentrations of 0.5 mM and 4 μM, respectively, to the cells 20 h later (roughly equivalent to one cell doubling time), according to previously described methods (33). Cell viability was monitored by absorption of MTT converted to formazan at 570 nm in an Enzyme 96-well plate reader ( Molecular Devices, Sunnyvale, CA).

**Tumor Treatment**—B16-F10 cells, cultured and maintained at ≤90% confluence, were suspended by trypsinization and washed once with complete media, resuspended in complete media and allowed to attach at 37 °C for 4 h, followed by 3 washes with 0.5% phenol red-free complete media. Sodium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) labeling reagent and N-methyl dibenzoazepine methyl sulfide (Sigma) electron coupling reagent were added at final concentrations of 0.5 mM and 4 μM, respectively, to the cells 20 h later (roughly equivalent to one cell doubling time), according to previously described methods (33). Cell viability was monitored byabsorption of MTT converted to formazan at 570 nm in an Enzyma 96-well plate reader ( Molecular Devices, Sunnyvale, CA).

**RESULTS**

**Cytosolic Delivery of Gelonin**—B16-F10 murine melanoma cells were treated with pH-sensitive liposomes containing LLO alone, gelonin alone, or both gelonin and LLO (GeLLOsomes). GeLLOsomes exhibited a dose-dependent cytotoxicity at lipid concentrations from 1.5 to 50 μM (Fig. 1). Effective cell killing resulting in the metabolic activity of cells below the limit of detection was achieved when cells were treated with GeLLOsomes at 100 μM lipid concentration. The IC₅₀ of the GeLLOsomes formulation was ~10 μM lipid or ~0.1 nM (~5 ng/ml) gelonin, as determined by SYPRO staining and Typhoon 9200 quantification of Sepharose CL-4B-purified GeLLOsomes—re
solved by SDS-PAGE (data not shown). The control formulations containing LLO alone or gelonin alone exhibited no detectable cytotoxicity (i.e. cell viability was statistically indistinguishable from that of untreated control cells). Cells pulsed with free (unencapsulated) gelonin exhibited no toxicity up to 3.3 \( \mu M \) (100 \( \mu g/ml \)) (data not shown), equivalent to the concentration of gelonin at the time of encapsulation in liposomes, and therefore a concentration that is ~3,300-fold higher than the highest effective dose (30 ng/ml gelonin and 100 \( \mu M \) lipid) of encapsulated gelonin in Fig. 1. Taken together, these data demonstrate the requirement of LLO co-encapsulation for efficient delivery of gelonin to the cytosol even with pH-sensitive liposomes.

**Dose-response of Gelonin**—To further investigate the dependence of cytotoxicity on gelonin concentration as well as estimate the minimum required concentration of gelonin for encapsulation, B16 melanoma cells were treated with GeLLOsomes containing a fixed concentration of LLO and varying concentrations of gelonin (Fig. 2). The data show that the cytotoxicity of the formulation was indeed dependent on the dose of entrapped gelonin. The formulations containing 100 \( \mu g/ml \) and 50 \( \mu g/ml \) encapsulation concentrations of gelonin exhibited statistically identical cytotoxicity with an IC\(_{50}\) of ~18 \( \mu M \) lipid for each, which corresponds to a concentration of ~1 nM gelonin incubated with cells. When the starting gelonin concentration was lowered to 10 \( \mu M \) lipid the IC\(_{50}\) increased to 10-fold higher. The cytotoxicity of GeLLOsomes containing 1 \( \mu g/ml \) encapsulated gelonin was below the detection limit (Fig. 2) with the short, 1 h incubation time utilized in this experiment.

**Effect of Pulse Time on Cytotoxicity**—pH-sensitive liposomal formulations have been reported to deliver protein to the cytosol in other systems (34–37). As we did not detect cytotoxicity, indicating presumably no significant cytosolic delivery, with a short incubation period of 1 h using pH-sensitive liposomes encapsulating gelonin alone, we postulated that a longer incubation time might enhance the net total amount of gelonin delivered to the cytosol. To test this hypothesis we pulsed B16 cells for 1, 8, or 24 h with liposome formulations containing gelonin alone, LLO alone, or GeLLOsomes, and monitored metabolic activity 24 h after pulsing (Fig. 3A). In this experiment the amount of liposomes used was equivalent to the highest dose used in previous experiments (i.e. 100 \( \mu M \) lipid and ~1 nM or ~30 ng/ml gelonin). As anticipated from the previous results, GeLLOsomes at this concentration exhibited 100% cell killing capacity at all three pulse times. In contrast, cells treated with formulations containing either gelonin alone or LLO alone exhibited no statistically significant cytotoxicity at pulse times of 1, 8, or 24 h.

**Cell Viability after Treatment**—The lack of detectable metabolic activity by XTT assay in the cells after treatment with GeLLOsomes can be interpreted as 100% cytotoxicity and cell killing. An alternative explanation, however, is that only a small number of cells have survived, and that is not sufficient after 24 h to be detectable by the XTT assay, whereas assaying at later time points after longer recovery and proliferation periods might allow detection above background of those cells that were not killed. To address these possibilities, B16 cells were treated with liposome formulations containing either LLO alone, gelonin alone, or both LLO and gelonin for 1, 4, or 8 h and their metabolic activity monitored by the XTT assay 1, 2, and 3 days after treatment. As shown in Fig. 3B, cells treated with GeLLOsomes at 100 \( \mu M \) lipid (~1 nM or 30 ng/ml gelonin) concentration for 1 h exhibited no significant level of metabolic activity after 3 days, whereas there was no significant reduction in the metabolic activity of cells treated with either the LLO alone or gelonin alone formulation. In this experiment, cells treated for 4 and 8 h were statistically indistinguishable from the corresponding 1-hour treatments; these data are therefore not shown for the sake of clarity (Fig. 3B). Morphological evidence of GeLLOsome cytotoxicity was obtained by examination of cell morphology using phase-contrast light microscopy, in which cells appeared rounded with few or no pseudopodia, whereas the morphology of cells treated with LLO alone or gelonin alone was indistinguishable from untreated cells (fibroblast-like, i.e. flattened with multiple extended processes (data not shown)). Taken together, these data support the notion that there is no delayed recovery observed in cells treated with the GeLLOsome formulation up to 72 h after pulsing.

**pH-sensitive, pH-insensitive, and PEGylated GeLLOsome Formulations**—The relative potency of the combined use of
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LLO and gelonin versus gelonin alone in pH-sensitive liposomes led us to test the hypothesis that LLO is able to significantly enhance the efficiency of delivering macromolecules even when used in combination with pH-insensitive liposomes (27, 34). To test this hypothesis B16 cells were treated with LLO and gelonin co-encapsulated in liposomes consisting of either PE:CHEMS (pH-sensitive) or PC:CHEMS (pH-insensitive) for 1, 8, or 24 h and their metabolic activity monitored by the XTT assay (Fig. 3C). Although the pH-insensitive GeLLO formulation exhibited reduced cell killing with a 1-hour treatment as compared with the pH-sensitive GeLLO liposomes (~43.2% viability versus 2.9%, respectively), longer treatment times resulted in the potency of the pH-insensitive formulation approaching that of the pH-sensitive (8 h treatment, 10.0 \textit{versus} 2.6%, respectively; 24 h treatment, 10.5 \textit{versus} 2.8%, respectively).

The limited circulation time of conventional liposomes has led to the development of “sterically stabilized” liposomes to reduce nonspecific uptake, particularly by the reticuloendothelial system, and consequently to improve circulation time (26, 27, 29, 30). The addition of PEG is one such modification that not only extends the \textit{in vivo} half-life of liposomes but one that also provides a molecular scaffold for the addition of targeting moieties (26, 28, 30, 38). With these considerations in mind we tested for cytotoxicity of our GeLLOsomes when 5 mole % of the PE was replaced with PEGylated PE (Fig. 3C). These data show that treatment of B16 cells for 1 h with PEGylated GeLLOsomes results in cell killing that is much less efficient than that by non-PEGylated GeLLOsomes and in cell viability that is statistically indistinguishable to that of untreated cells (86.3 \pm 15.3%). Viability of cells treated for 8 and 24 h, however, was reduced to 67.8 and 65.0%, respectively. These data are consistent with observations made with regard to the effect of PEGylation on liposome (both pH-sensitive and pH-insensitive) uptake by a variety of cells (39, 40). It therefore is likely that the reduced potency of our PEGylated GeLLOsome is due to its decreased uptake by B16 cells, although we have not directly tested this hypothesis and cannot rule out the possibility that the pH-sensitivity of the liposomes is more compromised upon incorporation of PEG-PE than that previously reported (39).

Effect of GeLLOsomes on Tumor Growth—As a first step toward assessing the relative \textit{in vivo} efficacy, as well as the LLO-dependence, of our gelonin-containing liposome formulations against tumor growth, we treated melanoma tumors formed subcutaneously in B6 mice by direct intratumor injection of free gelonin, or pH-sensitive liposomes containing gelonin alone or GeLLOsomes. The data indicate that the GeLLOsome formulation has the greatest relative potency for reducing the growth rate of these tumors (Fig. 4A). The tumor growth, shown as the average percent change in volume \pm S.D. from the beginning of treatment, was significantly reduced only with gelonin in LLO-liposomes relative to all other forms of treatment with gelonin. Comparison of treatment with liposomes containing gelonin alone (open circles) \textit{versus} GeLLOsomes (closed circles) on days where differences in percent change in tumor volume were qualitatively apparent (i.e. days 8–16) showed statistically significant differences in the two formulations (two-tailed \( p \) values \( \leq 0.02 \), except \( p = 0.1 \) on days 8 and 14), and on the last day of the experiment (day 16) there were no surviving mice from the control group. Survival of animals was also prolonged when treated with GeLLOsomes, as is apparent when the data are expressed on a per mouse basis (Fig. 4B). Average survival time of GeLLOsome-treated mice was \( 13.4 \pm 3.6 \) days \textit{versus} \( 9.2 \pm 3.5 \) days for those treated with gelonin encapsulated alone (two-tailed \( p \) value \( \leq 0.1 \)). This trend was reproduced in a second experiment in which groups of mice (\( n = 5 \) mice for treatment with GeLLOsomes or liposomes containing gelonin alone; \( n = 4 \) mice for treatment with unencapsulated gelonin or liposomes containing LLO alone) growing bilateral tumors were treated identically on both sides, with statistically significant differences (\( p \) values \( \leq 0.05 \) by the Mann-Whitney test) observed between GeLLOsome-treated and control-treated groups (data not shown). As was the case with cytotoxicity in the \textit{in vitro} studies, LLO-liposomes without gelonin did not show any detectable effect on tumor growth (data not shown). This is an important characteristic of LLO-
liposomes as cytosolic carriers in that the carrier itself is non-cytotoxic while it engenders efficient cytosolic delivery.

**DISCUSSION**

Efficient delivery across the plasma membrane is an important prerequisite to realizing the full potential of any therapeutic molecule whose target resides in the cytosol. In the case of macromolecules whose physical characteristics make accomplishing this task difficult or improbable, various strategies have been employed to try to circumvent the plasma membrane barrier or endosomal/lysosomal degradation. These strategies have included amphipathic cyclic or linear oligopeptides, cationic liposomes, viruses or virus-derived peptides, and polycationic polymers, all of which have some sort of membrane disrupting activities (41–47).

The approach to cytosolic delivery described here is one that mimics the cellular invasion strategy of the facultative intracellular bacterium *Listeria monocytogenes* (17, 18, 23). The same properties of LLO that make it well suited to promoting escape of *Listeria* from the degradative pathway into the cytosol of the host cell can be utilized to confer an advantage in the delivery of macromolecules in drug delivery systems. These properties include (1) the ability to form, in cholesterol-containing membranes, pores of sufficient size to allow passive diffusion of molecules at least $\sim 45$ kDa (19, 20, 22, 48, 49) and to cause endosomal vesicle lysis or rupture, (2) a pH-dependent activity that is highest at the pH of the endosome lumen (pH 5.5–5.9), making it relatively less active against membranes such as the plasma membrane in a neutral pH environment (50–52), and (3) an N-terminal PEST-like sequence that facilitates rapid LLO degradation within the cytosol via the ubiquitin/proteasome pathway (53, 54), making it even less likely that LLO would damage other cellular membranes subsequent to its release from the endosome.

We have demonstrated in this report the advantage of using LLO in pH-sensitive liposomes to facilitate delivery of a membrane-impermeant functional protein to the cytosol of B16-F10 murine melanoma cells. Gelonin, a type I ribosome inactivating plant-derived toxin, has been shown to be a potent inhibitor of cell growth, but one that is relatively inefficient in gaining entry to the cytosol (4). Because of this latter property, one major benefit to using gelonin is its relatively low toxicity when applied extracellularly (4). This general property is consistent with our experiments in which free (unencapsulated) gelonin showed no detectable cell-growth inhibition when incubated with B16 cells at 100 $\mu$g/ml, the concentration of gelonin used for encapsulation in liposomes. We were also unable to detect toxicity with gelonin encapsulated alone in pH-sensitive liposomes (Fig. 1). These data suggest that even under conditions in which gelonin was likely delivered to the endosome, insufficient quantities were able to gain entry to the cytosol to exert a measurable effect of cell growth inhibition or cell killing. That gelonin is likely released into the endosome in the absence of LLO is supported by a variety of studies that utilize the PE-CHEMS formulation for pH-sensitive liposomes (27, 34). Although not directly comparable with other studies employing pH-sensitive liposomes, we infer that the lack of detectable cytotoxicity, and therefore lack of cytosolic delivery, of our PE-CHEMS/gelonin formulation may be a manifestation of parameters not directly related to the inherent efficiency of release from pH-sensitive liposomes (e.g. encapsulation efficiency, stability of the protein during liposome formation and/or in the endosomal/lysosomal pathway, differences in endocytic rates between cell types, sensitivity of the assay, etc.). It is interesting that we were able to observe substantial toxicity (90%) with LLO and gelonin in pH-insensitive liposomes when cells were treated for extended periods (Fig. 3B), even though treatment of cells for the same time periods with gelonin encapsulated in pH-sensitive liposomes without LLO resulted in no detectable cytotoxicity (Fig. 3A). Taken together, these results suggest that although the pH-sensitivity of liposomes composed of PE-CHEMS is an elective component aiding the achievement of the full efficacy of our GelLOsome formulation, it is nonetheless insufficient in the absence of LLO.

Prior gelonin formulations were shown to enhance cytotox-
icity by incorporating targeting motifs such as hormones, antibodies, growth factors, and vitamins, as well as by packaging gelonin with endosomolytic polymers or within liposomes (5–11, 14, 55). To our knowledge, the most potent gelonin formulations tested to date were targeted ones with IC_{50} values approximating our calculated IC_{50} value of 0.1 nM gelonin in the GeLLOsome formulation (11, 14, 56). It is worth noting that by including LLO we were able to achieve this gelonin IC_{50} without the addition of a targeting motif. Particularly noteworthy, moreover, is that with the non-targeted GeLLOsome formulation we were able to achieve a similar gelonin IC_{50} value with an incubation time of only 1 h, versus 24 h (56) or 72 h (11, 14) for the targeted formulations. This potency is likely a reflection of two distinct yet complementary phenomena. First, the use of liposomes allows for an increase in the effective concentration of drug at the cell surface. That which is encapsulated would be relatively protected from degradation prior to reaching the target, and each liposome-binding event would deliver an amount of cargo commensurate with its encapsulation efficiency. Second, the ability of LLO to perforate the endosome membrane upon release from the pH-sensitive liposomes allows for a greater fraction of gelonin to escape intact into the cytosol than is otherwise achievable.

We estimate that there are ~15–20 gelonin molecules encapsulated per liposome in our GeLLOsome formulation (see “Experimental Procedures”). Because it has been shown that one or a few molecules of similar toxins are capable of killing cells when cytoplasmically injected (3, 57, 58), one could conclude that despite the relative effectiveness of the GeLLOsome delivery system, we still have encountered some barriers to achieving an “ideal” potency. These obstacles might include degradation or inactivation of gelonin or LLO during liposome preparation, stability of the PE:CHEMS liposome formulation in cell culture media, degradation within the endosomal/lysosomal pathway, or relatively inefficient uptake of the liposomes by B16 cells. We would therefore predict that by incorporating a targeting motif on the surface of stERICally stabilized GeLLOsomes we would achieve even greater potency than that observed with our non-targeted formulation.

The utility of GeLLOsomes, tested using a cell culture system as in Figs. 1–3, was also monitored in preliminary, proof-of-concept in vivo experiments by intratumor injection into B16 cells growing subcutaneously as a solid tumor mass in a mouse melanoma model. The direct injection route was chosen because the PEGylated GeLLOsome formulation was ineffective in the cell culture system; without the long circulation provided by PEGylation, the GeLLOsome formulation has little chance to reach and accumulate in the solid tumors. In this context, the GeLLOsome formulation tested in this report was prepared similarly, including the final size of liposomes used, to those formulations observed in vitro and strongly support the advantage of LLO-mediated cytosolic delivery of gelonin.

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