Targeted cytolysins synergistically potentiate cytoplasmic delivery of gelonin immunotoxin

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Supplementary Information

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Methods

S1. Construction of expression plasmids. Genes encoding the LLO and PFO proteins were codon-optimized for expression in *E. coli* and ordered from GenScript (Piscataway, NJ). Genes were amplified by PCR using primers designed with 3' complimentarity to either end. Gene products were inserted into the pMal fusion construct used previously for immunotoxin synthesis by the method of Geiser *et al.*[1,2]. The resulting plasmid encoded an open reading frame including, from N-terminus to C-terminus: maltose binding protein, N_{10} linker, Factor Xa protease site, an engineered 10th Type III fibronectin domain, a G_4S linker, and LLO or PFO. Additionally, our PFO gene was truncated to remove an unnecessary secretory sequence and its construct modified to incorporated a tobacco-etch virus protease site N-terminal to the constant Factor Xa site.

S2. Protein expression and purification. Immunotoxins were synthesized as described previously [1] and Fn3-LLO/PFO production was conducted in a similar manner. Briefly, the appropriate plasmids were transformed into Rosetta (DE3) *E. coli* (Novagen, San Diego, CA). Isolated colonies were used to inoculate 15 mL of selective media incubated overnight at 37 °C. Seed cultures were used to inoculate 1 L of antibiotic-free, rich LB media and grown to an OD_{600} ~ 0.5 before IPTG induction at 30 °C for 6 hours. Cultures were then centrifuged at 15,000 x g and the cell pellets frozen at -20 °C. Pellets were resuspended in 25mL amylose column buffer containing Complete EDTA-free protease inhibitor (Roche, Indianapolis, IN) and then sonicated on a Branson Sonifier 450A (Branson Ultrasonics, Danbury, CT) at 50% duty cycle and power level 6 for three, one-minute intervals. The resulting solution was centrifuge at 50,000 x g and supernatant was applied to an amylose column as described by the manufacturer (New England

Biolabs, Ipswich, MA). Purified recombinant proteins were concentrated and buffer exchanged into Factor Xa digestion buffer and incubated overnight at 4°C with Factor Xa (New England Biolabs). Fn3-cytolysin was isolated by ion-exchange chromatography with a HiTrap Q column (GE Healthcare, Piscataway, NJ). Larger scale protein production was conducted using the same protocol executed at 2L and 10L scales in Bioflo bioreactors (New Brunswick Scientific, Edison, NJ) with oxygen control supplied by filtered air at 0.5 (vvm) and agitation adjusted to maintain dissolved oxygen levels above 30%. pH was controlled at 7.0 using 6N NaOH. Cells were grown to OD600 = 0.8 before induction as before.

S3. Antigen binding affinity titration. Fn3-LLO was biotinylated using amine reactive EZ-Link Sulfo-NHS-LC-biotin (Pierce, Rockford, IL). Antigen positive cell lines HT-1080(CEA) and A431 were lifted from culture plates and resuspended in 4% formalin for 30 minutes. Fixed cells were incubated with varying concentrations of biotinylated fusion protein overnight at 37 °C, washed once and resuspended in 250 μL of PBSA with 1 μL streptavidin-phycoerythrin conjugate (Sigma, St. Louis, MO) for 1 hour at 4 °C. Cells were washedagain and resuspended in 150 μL PBSA before analysis on an Accuri C6 Flow Cytometer (Accuri Cytometers, Ann Arbor, MI). For each titrating concentration, the median fluorescent intensity was determined and data sets for each immunotoxin were fitted to a standard binding isotherm using least-squares regression.

To titrate Fn3-PFO fusions, CEA (R&D Systems, Minneapolis, MN) was first biotinylatedand then loaded on streptavidin coated magnetic beads (Invitrogen, Carlsbad, CA), and incubated with C7PFO for 6 hours at 4 °C. EGFR 404SG ectodomain was expressed on the surface of yeast[3], and incubated with E6PFO for 6 hours at 4 °C. Cells or beads were washed

once and resuspended in 50 μL of PBSA and incubated for 30 minutes at 4 °C with 0.25 μL of rabbit anti-His6 antibody (Abcam, Cambridge, MA) which was labeled with the Alexa 647 Microscale Protein Labeling Kit (Invitrogen). Cells or beads were washed twice with 200 μL PBSA before analysis by flow cytometry.

S4. Hemolysis. Experiments were conducted using a method similar to that described by Henry *et al*. [4]. Briefly, purified mouse red blood cells (Fitzgerald, Acton, MA) were washed twice with PBSA at either pH 7.4 or pH 5. Cells were mixed with fusion protein in the same buffer to a final cell concentration of ~$1x10^9$ cells/ml. Cells and protein were incubated at 37 °C for 1 hour before treatments were centrifuged at 13,500 x g for 5 minutes. Supernatants were transferred to 96-well plates and absorbance read at 540 nm. Results were normalized to a PBS negative control and 1% Triton-X100 positive control.

S5. Cytotoxicity. Log-phase tumor cells were counted and seeded on 96-well plates at 2,500 cells per well. Cells were allowed to adhere overnight, after which fresh growth media containing Fn3-cytolysin and/or immunotoxin was added to triplicate wells. Cells were incubated in treatment media for 48 hours before being replaced with media containing the WST I reagent (Roche). The red/ox solution was allowed to develop for 1 hour under normal culture conditions after which plates were measured for absorbance at 450 nm. Untreated cells and cells lysed with a 1% Triton-X100 solution were used as positive and negative controls, respectively. Measurements were set to baseline on negative control and normalized to positive control treatments. Delayed dose or time dependent cytotoxicity data were obtained by treating cells as described, removing treatment containing media, washing once with PBS, then replacing with

fresh media or media containing the second agent for wells at each time point then following identical assay procedures after 48 hours. In combination treatments where one agent was titrated and the other was fixed or where both agents' concentrations were fixed, the fixed concentration was selected so as to be non-toxic in the absence of the other agent.

S6. D**ose escalation.** Dose escalation was carried out for all *in vivo* applied fusion proteins using the canonical "3+3 method"[5] in which 3 mice are dosed at a particular concentration and if no dose limiting toxicity is observed then the dose is raised, if one of three mice exhibit limiting toxicity then a new cohort of three mice are treated at the same dose, and if two or more mice show limiting toxicity then escalation is stopped and the previous dose is deemed the maximum tolerated dose. Here, in the absence of any *a priori* knowledge on which to base a starting dose, a logarithmically spaced escalation was employed where after limiting toxicity was observed a linear escalation was continued from the last tolerated dose.

S7. Plasma clearance. Fusion proteins were labeled with Li-Cor 800CW dye(Li-Cor Biosciences, Lincoln, NE). Labeled proteins were injected at their respective maximum tolerated doses into three mice and blood samples taken at logarithmically spaced time points by tail clipping. Blood was collected into heparin coated capillary tubes and image on a Li-Cor Odyssey Imaging System (LiCor Biosciences). Imaging sensitivity was adjusted to maximize signal-to-noise ratio without saturating the fluorescence channel. Fluorescent signals were averaged across mice at each time point and fitted with a bi-exponential function for retro-orbital injections and with a tri-exponential for intraperitoneal injections to determining absorption and clearance constants as well as plasma half-lives.

S8. Dose separation. Using clearance information as a basis we applied the same "3+3 method" as for dose escalation to determine the minimum separation time between doses. Gelonin immunotoxin was dosed first by retro-orbital injection at its independent maximum tolerated dose. After the defined amount of time targeted cytolysin was dosed at its own independent maximum tolerated dose either by retro-orbital injection in the opposite eye or by intraperitoneal injection. Mice were monitored for toxicity for 72 hours following the second injection.

S9. *in vivo* model system. All *in vivo* studies used 6-8 week old athymic N_{cr} (nu/nu) nude mice obtained from Taconic (Hudson, NY). This is the standard model for National Cancer Institute studies and many pharmaceutical and oncology screening programs. Their outbred background originates from BALB/c and NIH(s) stocks.

Supplementary Methods References

1. Pirie, C. M., Hackel, B. J., Rosenblum, M. G. & Wittrup, K. D. Convergent potency of internalized gelonin immunotoxins across varied cell lines, antigens, and targeting moieties. *J. Biol. Chem* **286**, 4165–4172 (2011).
2. Geiser, M., Cèbe, R., Drewello, D. & Schmitz, R. Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. *BioTechniques* **31**, 88–90, 92 (2001).
3. Kim, Y.-S., Bhandari, R., Cochran, J. R., Kuriyan, J. & Wittrup, K. D. Directed evolution of the epidermal growth factor receptor extracellular domain for expression in yeast. *Proteins* **62**, 1026–1035 (2006).
4. Henry, S. M., El-Sayed, M. E. H., Pirie, C. M., Hoffman, A. S. & Stayton, P. S. pH-responsive poly(styrene-alt-maleic anhydride) alkylamide copolymers for intracellular drug delivery. *Biomacromolecules* **7**, 2407–2414 (2006).
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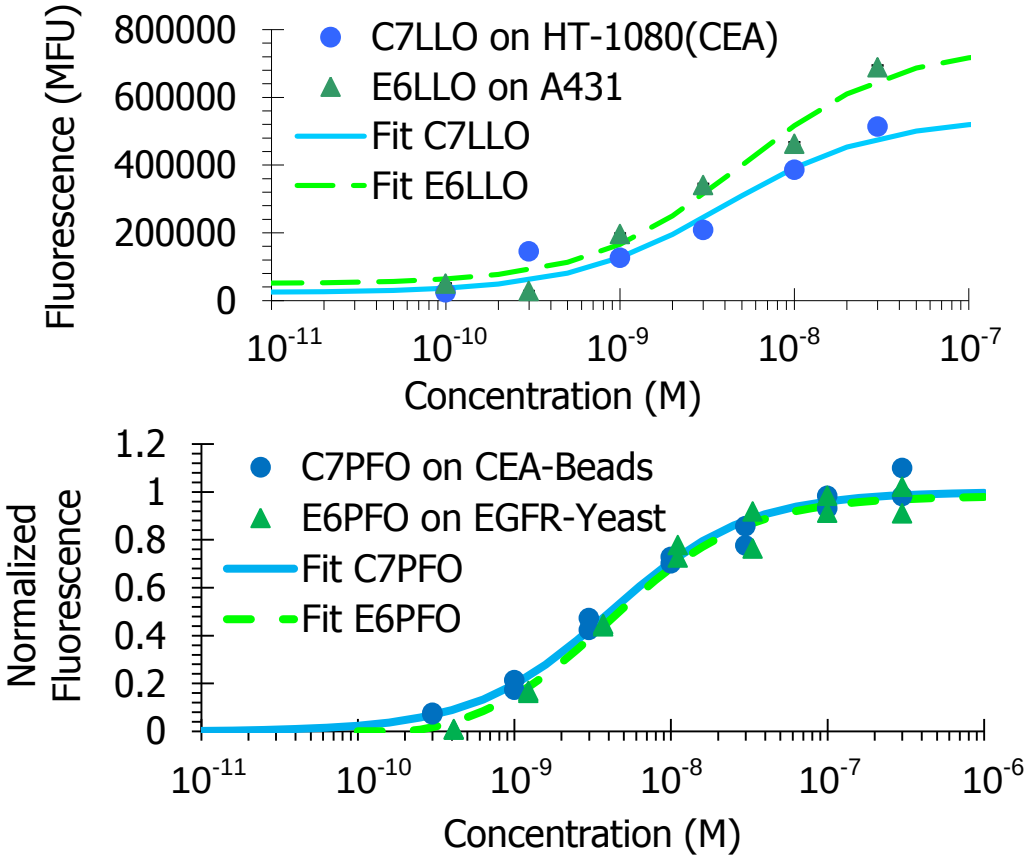
Figures

S1.

a

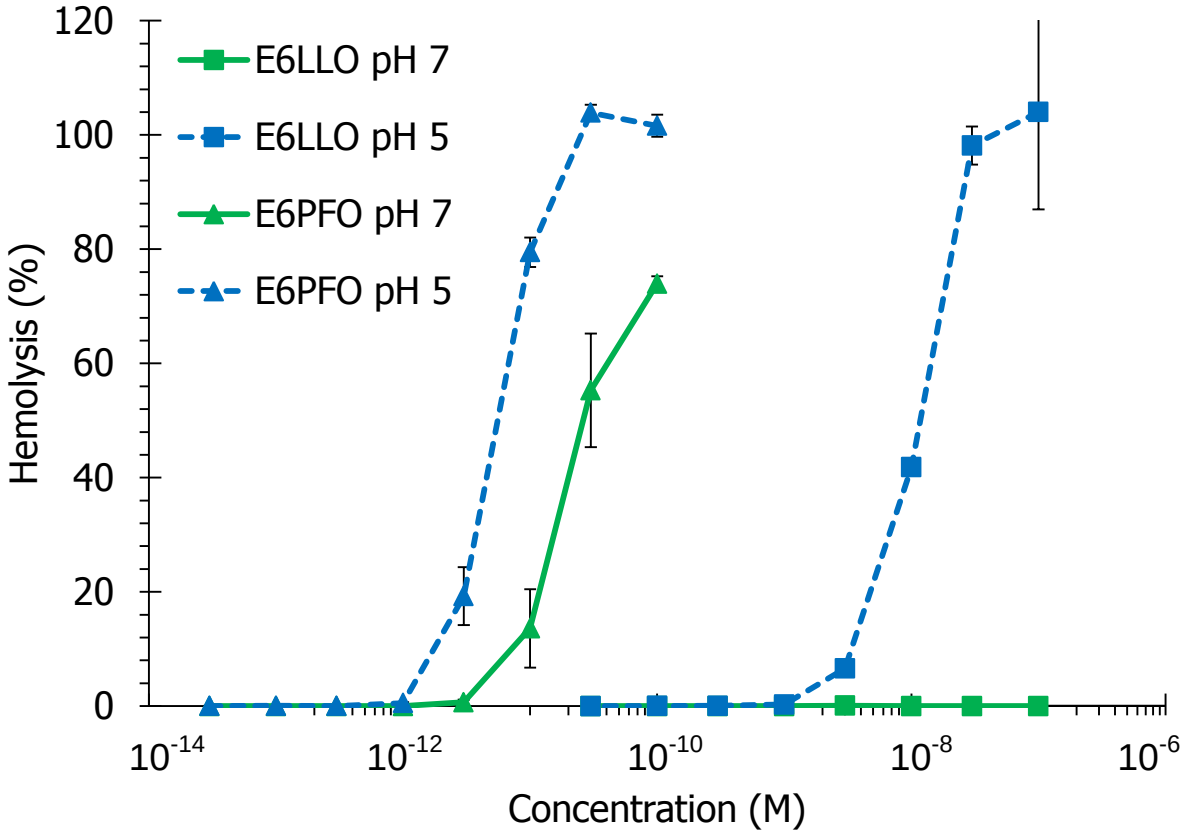


b

IC_{50}'s (M)	*Cell Line*			
Potentiator	HT-1080	HT-1080 (CEA)	A431	HT-29
C7LLO	$>3x10^{-7}$	$8.3x10^{-8}$	-	$>1x10^{-6}$
E6LLO	$2.2x10^{-7}$	$2.5x10^{-7}$	$7.1x10^{-8}$	$>1x10^{-6}$
C7PFO	-	$7x10^{-12}$	$9.8x10^{-12}$	$1.2x10^{-10}$
E6PFO	-	$1x10^{-11}$	$5.4x10^{-10}$	$1.5x10^{-11}$

Supplementary Figure S1 Potentiator binding and cytotoxicity. **(a)** Biotinylated Fn3-LLO fusions were titrated against fixed A431 or HT-1080(CEA) cells. Cells were washed an incubated for 1hr on ice with streptavidin-phycoerythrin and then assayed by flow cytometry. His6 tagged PFO fusions were titrated against CEA-coated beads or EGFR-displaying yeast and labeled with an anti-His6 antibody. Fusions showed binding affinity K_d's in the low nM range, only slightly reduced relative to the parent Fn3 affinity. **(b)** Fusions were added to growth media on cells with varying antigen expression levels, in the absence of any immunotoxin. For each cell type, Fn3-cytolysin cytotoxicity correlated with antigen expression level.

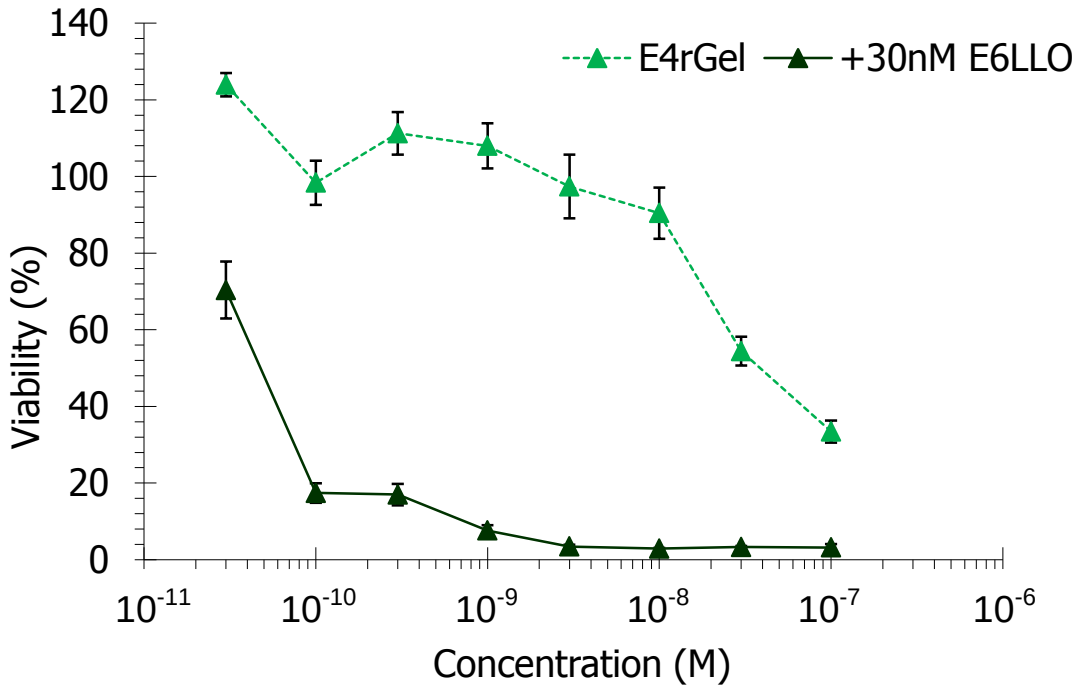
S2.



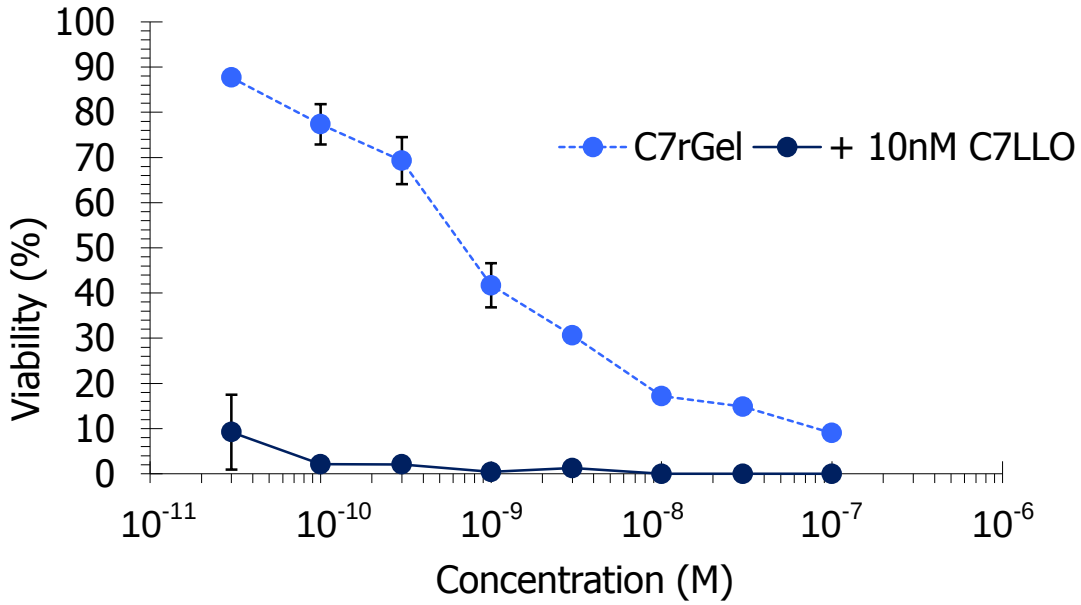
Supplementary Figure S2 Potentiator hemolytic activity. Fn3-cytolysins were incubated with mouse red blood cells at either physiological (7) or endosomal pH (5). Fusions showed the expected pH dependent response at concentrations consistent with their non-specific toxicity and potentiating activity.

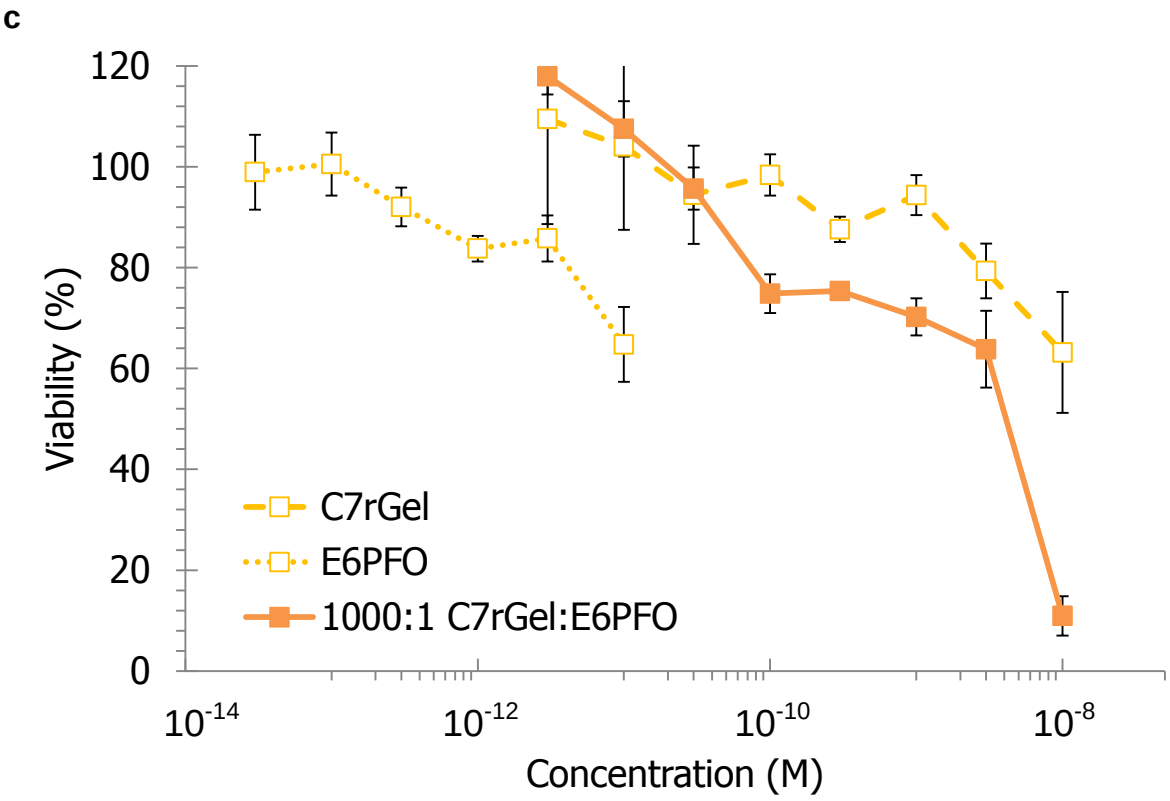
S3.

a


E4rGel
+30nM E6LLO
Viability (%)
Concentration (M)

b

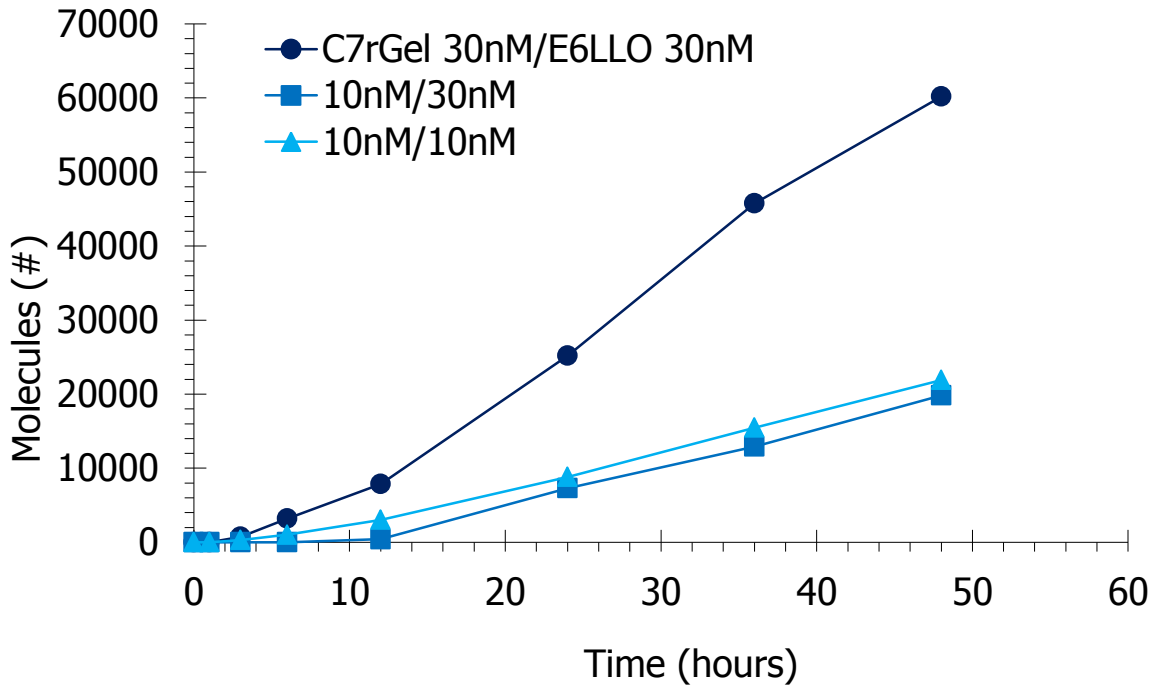

C7rGel
+ 10nM C7LLO
Viability (%)
Concentration (M)



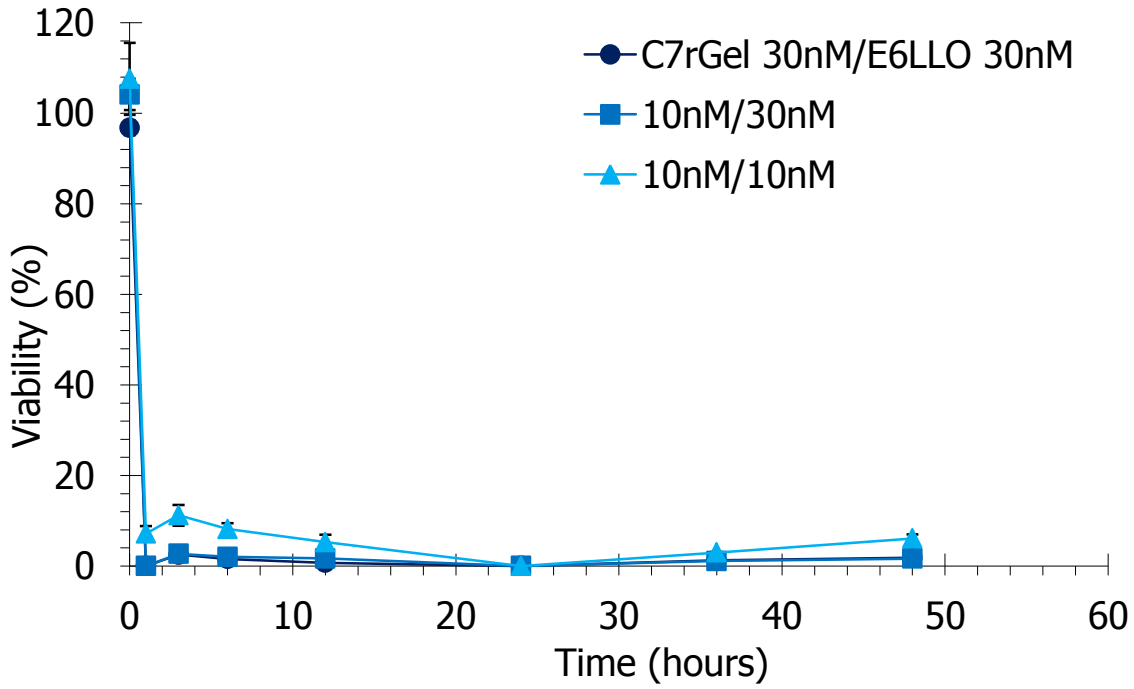
Supplementary Figure S3 Potentiation of gelonin immunotoxin cytotoxicity. Gelonin immunotoxins were titrated on high antigen expressing cell lines in the absence or presence of non-toxic concentrations of targeted listeriolysin fusions. **(a)** Potentiation of anti-EGFR immunotoxin E4rGel using the non-competitive, co-targeted potentiator E6LLO on A431 cells. **(b)** anti-CEA immunotoxin C7rGel potentiated by the competitive potentiator C7LLO on HT-1080(CEA) cells. **(c)** Titrations of C7rGel and E6PFO on HT-29 cells achieve only modest toxicity alone, but when titrated together at a ratio of 1000 C7rGel:1 E6PFO much greater toxicity is observed.

S4.

a


70000
60000
50000
40000
30000
20000
10000
0
Molecules (#)
0
10
20
30
40
50
60
Time (hours)
C7rGel 30nM/E6LLO 30nM
10nM/30nM
10nM/10nM

b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80
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40
20
0
Viability (%)
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10
20
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40
50
60
Time (hours)
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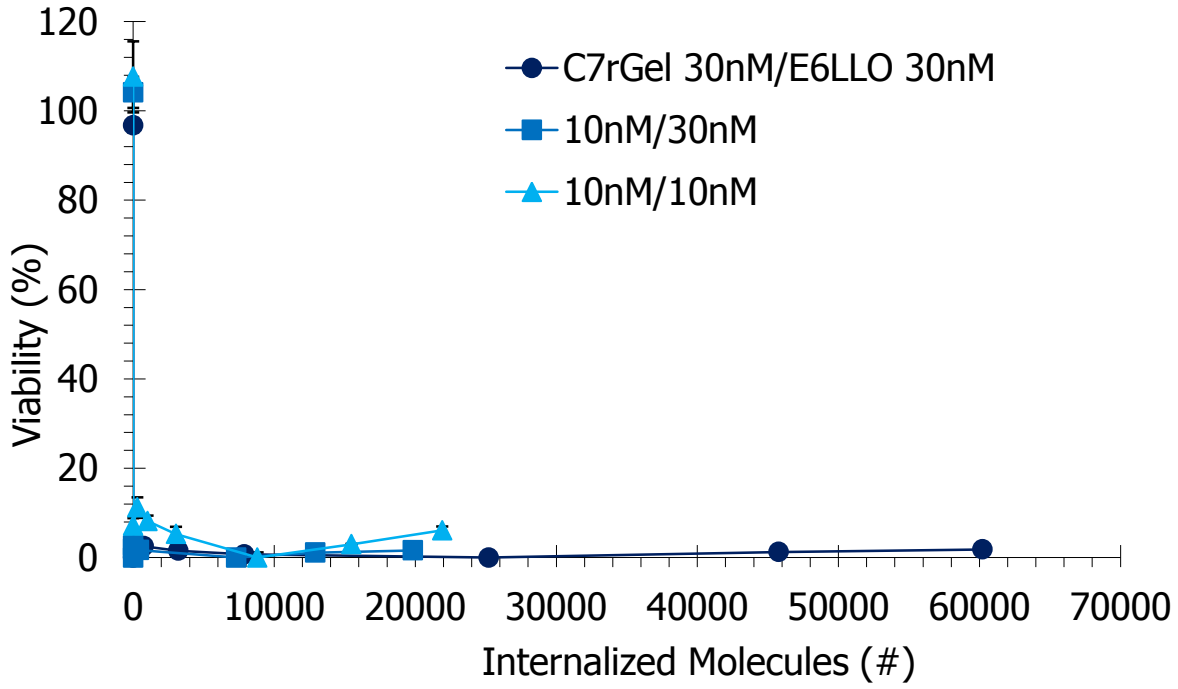
10nM/30nM
10nM/10nM

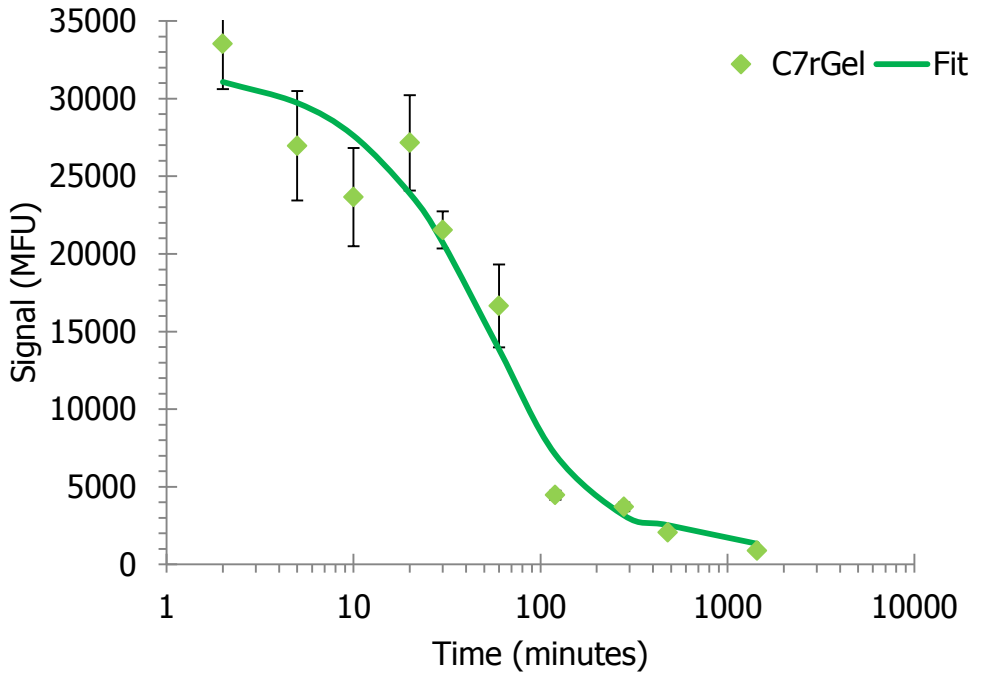
c



Supplementary Figure S4 Potentiaion of internalized cytotoxicity and reduction of gelonin immunotoxin TN_{50}. Immunotoxins were fluorescently labeled and their internalization by antigen positive cells was measured and quantified for the precise number of toxins inside. In the absence of potentiator, ~$5x10^6$ toxins must be internalized on average before a cell will undergo apoptosis. **(a)** HT-29 cells treated with independently non-toxic levels of immunotoxin which shows concentration and time dependent internalization in the presence of potentiator. **(b)** HT-29 cells treated with equivalent doses show concentration and time dependent loss of viability. **(c)** Combined data sets comparing loss of viability with respect to uptake indicates that fewer than $5x10^3$ immunotoxins were sufficient to induce apoptosis

S5.

a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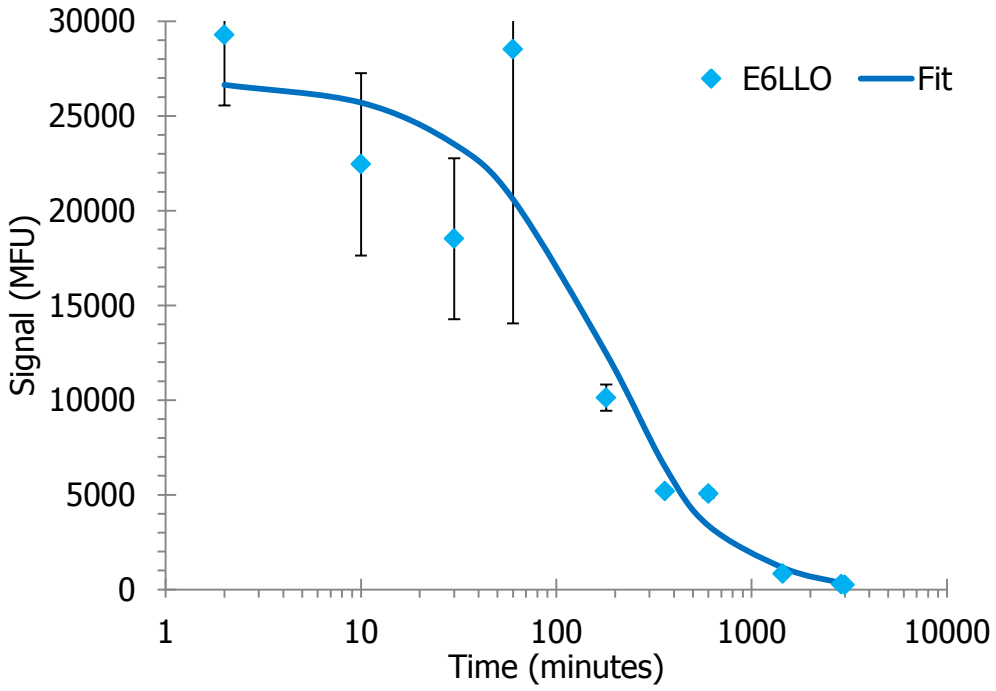
30000
25000
20000
15000
10000
5000
0
Signal (MFU)
1
10
100
1000
10000
Time (minutes)
C7rGel
Fit

b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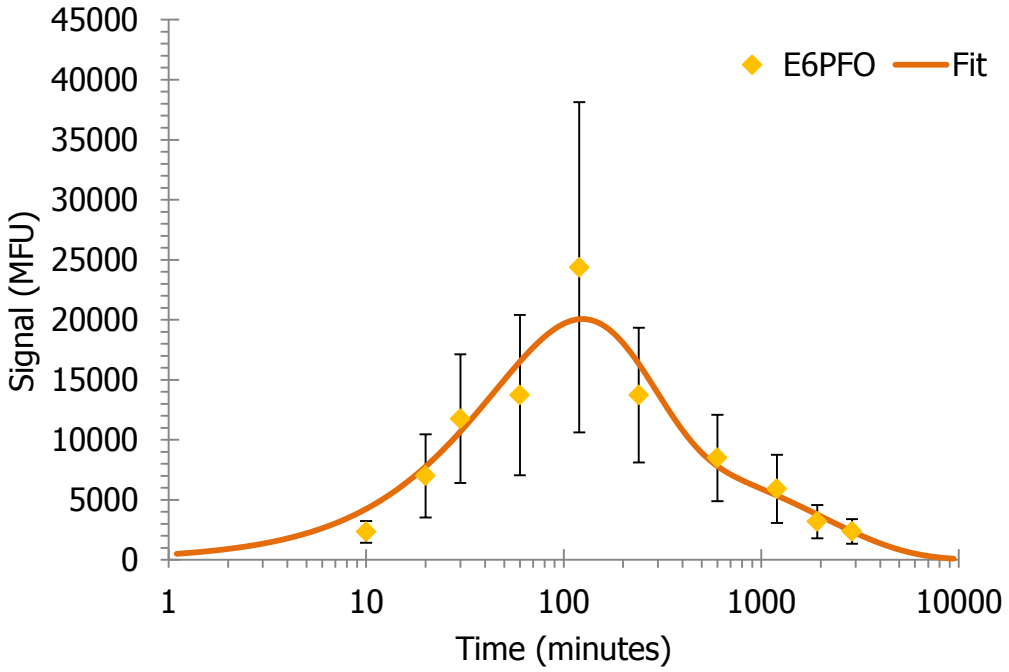
Time (minutes)
E6LLO
Fit

c



Supplementary Figure S5 Plasma clearance of immunotoxin and potentiators. Proteins used for *in vivo* testing were labeled with infra-red dye and injected into groups of three mice. Blood samples were collected at logarithmically spaced time points from the tail. **(a)** C7rGel injected retro-orbitally. **(b)** E6LLO injected retro-orbitally. **(c)** E6PFO injected intraperitoneally.

Tables

S1

C7rGel Dose (mg/kg)	Morbidity	E6LLO Dose (mg/kg)	Morbidity	E6PFO Dose (mg/kg)	Morbidity
0.2	0/3	0.02	0/3	0.02	0/3
0.8	0/3	0.08	0/3	0.08	0/3
2	0/3	0.2	0/3	0.2	0/3
8	0/3	0.8	2/3	0.8	3/3
12	0/3	0.4	0/3	0.4	2/3
16	0/3	0.6	0/3	-	-

Supplementary Table S1 Independent dose escalation of therapeutic proteins. The "3+3 method" was used to assess dose limiting toxicities as each agent to be used for *in vivo* testing was administered at increasing levels. We found that for C7rGel substantial doses could be given without toxicity but that for E6LLO and E6PFO the maximum tolerated dose was less than 1 mg/kg. Doses used for plasma clearance, minimum dose separation time, and tumor growth inhibition studies as maximum tolerated dose are boxed.

S2.

	Dose Separation Time (hours) – Morbidity					
RO C7rGel + RO E6LLO	24 – 0/3	18 – 0/3	12 – 0/3	6 – 3/3	-	-
RO C7rGel + RO E6PFO	24 – 0/3	18 – 2/3	-	-	-	-
RO C7rGel + IP E6LLO	-	-	-	6 – 0/3	3 – 1/3	-
RO C7rGel + IP E6PFO	24 – 0/3	18 – 0/3	12 – 0/3	6 – 0/3	3 – 0/3	0 – 2/3

RO = Retro-orbital
IP = Intraperitoneal

Supplementary Table S2 Determination of the minimum delay between *in vivo* doses of synergistic agents. When administering by retro-orbital injection which results in an almost instantaneous maximum plasma concentration we found that subsequent doses required at least a 12 hour delay. Alternatively when we administered secondary injections of targeted cytolysins into the peritoneal cavity where absorption into the plasma was slowed, we found that doses could be given with as little as 3 hours delay.