Methods

Materials. Tetrodotoxin (TTX) was obtained from Abcam plc (Cambridge, MA); Dexmedetomidine hydrochloride (DMED) was acquired from R&D systems, Inc. (Minneapolis, MN). DPPC and DPPG were acquired from CordenPharma International (Plankstadt, Germany). Cholesterol and rhodamine 6G were purchased from Sigma (St, Louis, MO). Thiolated poly(ethyl glycol)-1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine (HS-PEG-DSPE, Mw 2000 Da) was from NanoCS Inc. (New York, NY). Tetrodotoxin ELISA kits were purchased from Reagen LLC (Moorestown, NJ). Gold nanorods were synthesized as previously reported.[1] PEGylation of GNRs was conducted by incubating with methoxy-PEG-thiol (Mw 2000 Da, Laysan Bio, Arab, AL) for 24 hours at room temperature and dialysis against deionized water for three days.[2]

Preparation of Lip-GNRs. A lipid cake was produced as previously described.[3] DPPC, DPPG, cholesterol and HS-PEG-DSPE (molar ratio 6:2:3:0.2) were dissolved in a chloroform:methanol (v/v, 9:1) mixture and formed a lipid bilayer under reduced pressure. The bilayer was hydrated with tert-butanol and vacuum-dried to form a fluffy liposome cake. Then the cake was hydrated with 0.25 M ammonium sulfate solution containing GNR (0.04 wt%). The Lip-GNR-0 were homogenized at 10,000 rpm with a 3/8" Mini-Micro workhead on a L4RT-A Silverson Laboratory Mixer (East Longmeadow, MA) for 10 minutes, then treated with 10 freeze-thaw cycles and dialysis (MWCO 50 KDa, Float-A-Lyzer®G2, Spectrum Laboratories, Inc., Rancho Dominguez, CA) against PBS (6 changes of buffer over three days). The Lip-GNR-0 solution was centrifuged at 1000×g for 10 minutes to remove any free GNR. The purified Lip-GNR-0 were characterized by particle analyzer (Delsa Nano C, Beckman Counter) and imaged with cryo-EM (JEOL 2100). The concentration of gold was analyzed by inductively coupled plasma mass spectrometry (ICP-MS, Sciex Elan 6100, Perkin Elmer, Norwalk, CT). To prepare Lip-GNRs loaded with drugs or dye, the same procedure was followed with addition of TTX (0.5 mg/mL) and DMED (0.3 mg/mL), or R6G (2.8 mg/mL) in the 0.25 M ammonium sulfate hydration buffer. To form liposomes without GNRs, the same procedure was followed except with 0.25 M ammonium sulfate without GNRs. Drugs and dye in all formulations were quantitated after disruption of the liposome with octyl-β-D-glucopyranoside (100 mM, volume ratio of 2:1 to formulations). The concentration of R6G was determined by a plate reader (BioTek, Winooski, VT) with excitation and emission wavelengths of 535 nm and 580 nm. To study the

self-quenching effect of R6G in liposome, 0.4 mL of Lip-GNR-R6G were incubated at 4, 37 and 43^{o}C. At each predetermined time point, 10 μL of Lip-GNR-R6G was withdrawn and the fluorescence intensity was determined after 200 folds dilution with PBS. DMED and TTX were quantitated by HPLC (at 215 nm, Agilent 1260 series system) and ELISA, respectively. The concentration of lipid was determined by the Bartlett assay as previously reported.[4, 5]

To study the effect of temperature on R6G release, 0.4 mL of Lip-R6G or Lip-GNR-R6G was placed in a dialysis device (Slide-A-Lyzer® MINI, MWCO 20 KDa, Thermo Fisher Scientific Inc, Grand Island, NY) against 13.5 mL PBS. At each predetermined time point, the buffer was changed with fresh PBS. The release of TTX and DMED from Lip-GNR-TD at 37 ^{o}C was studied and quantitated by ELISA and HPLC.

Photosensitivity of Lip-GNRs. To assess the photosensitivity, Lip-GNR-0 (0.1 mL) were placed in a 96-well plate and irradiated with an 808 nm continuous wavelength (CW) NIR laser for 1-30 minutes, with irradiances ranging from 8 to 75 mW/cm^2. The temperature was detected using an FLIR E50 infrared imaging camera (FLIR Systems, Wilsonville, OR) at the beginning and end of each cycle of irradiation. To detect the stability of Lip-GNR-0 after multiple triggers, Lip-GNR-0 were repeatedly irradiated with the NIR laser at 75 mW/cm^2 for 15 minutes, followed by cooling at RT for 30 minutes. The size was characterized with particle analyzer at the end of each off-state.

Phototriggered release in vitro. The phototriggered release of R6G was evaluated by measuring the fluorescence intensity. Lip-GNR-R6G (0.1 mL) was placed in a 96-well plate and irradiated with an 808 nm CW NIR laser for 10 minutes. The samples were diluted 200 folds with PBS and the fluorescence intensity was measured with a plate reader (BioTek, Winooski, VT). The release percentage of R6G was quantitated based on a standard curve that was normalized to 100% dye release obtained by bursting the liposomes with 100 mM octyl β-D-glucopyranoside (2:1 volume ratio to Lip-GNR-R6G). To evaluate repeated triggered release of R6G, 100 μL of Lip-GNR-R6G were irradiated at 17 mW/cm^2 for ten minutes, followed by cooling at RT for 30 minutes. 5 μL of sample was withdrawn for determination of R6G release. The remaining solution was centrifuged at 4000×g for 10 minutes and the supernatant was changed with PBS. Lip-GNR-TD were dialyzed against PBS at 37 ^{o}C for 24 hours to remove the burst release of TTX and DMED. Phototriggered release of TTX and DMED from Lip-GNR-TD was assessed by ELISA and HPLC, respectively. To evaluate multiple triggered release, Lip-GNR-TD were irradiated with NIR laser for 10 minutes for five

cycles separated by 30 minutes. Free drugs after each trigger were removed by centrifugation (4000×g, 10 minutes) and the Lip-GNR-TD were re-suspended in fresh PBS.

Cytotoxicity. C2C12 mouse myoblasts (ATCC, CRL-1772) were cultured in DMEM supplemented with 20% FBS and 1% Penicillin Streptomycin. Cells were seeded into 96-well plate at 50,000 cells per mL in DMEM with 2% horse serum and 1% Penicillin/Streptomycin, and cultured for 10-14 days until the cells differentiated into myotubes. During that time, media were changed every 3-4 days. PC12 cells (ATCC, CRL-1721) originating from rat adrenal gland pheochromocytoma were plated in a 24-well plate at the density of 20,000 cells/well, with F-12K supplemented with 12.5% horse serum, 2.5% FBS, and 1% Penicillin/Streptomycin. 24 hours after seeding, the culture medium was changed to 1% horse serum, 1% Penicillin/Streptomycin and 50 ng/mL nerve growth factor (Life technologies, Grand Island, NY). Media were changed at day 4 and the cells were cultured up to 7 days. The formed myotubes and differentiated PC12 cells were incubated with 100 µL Lip-GNR-TD placed in an insert with porous membrane (0.4 µm). Cell viability was assessed with an MTS assay kit (Promega Corporation, Madison, WI).

Phototriggered local anesthesia in vivo. Lip-GNR-TD were mixed with Lip-GNR-R6G with a volume ratio of 9:1. Animals were handled daily in a quiet room for up to 7 days before the treatments to familiarize them with the behavioral investigator, the experimental environment, and the specific experimental procedures. The mixture (100 µL) was injected into the footpad of the rat under isoflurane-oxygen anesthesia. Nociceptive behavioral test was conducted by recording the audible vocalization as previous reports with modification.[6-9] The behavior investigator was not aware of which anesthetic treatment was assigned to any given rat (i.e., he was “blinded”). We measured the degree of infiltration anesthesia by poking the rat footpad with Touch Test® sensory evaluators (North coast medical, Inc., Gilory, CA), a series of filaments which provide a defined force (although expressed in gram units) when applied to the body surface. We assessed the force that elicited a vocal or motor (foot withdrawal) response from the animal when we poked the footpad. The greater the force required to elicit a response, the more intense (“dense”) the local anesthesia. Filaments with a target force of 26 g, 60 g, 100 g, 180 g and 300 g were used. Forces above 300 g were not used, in order to avoid damage to the footpad[10]. In the absence of local anesthetic treatments, none of rats responded to the filament with 26 g target force. They began to respond to filaments with a target force of 60-100g, and all responded to a filament with 180 g target force.[8] Consequently, 180 g

was the force used in testing animals. Each rat was tested five times at each predetermined time point. The effectiveness of local anesthesia was expressed as the percentage (number of times out of 5 x 100) that a rat did not respond (vocalize and/or withdraw) in response to 180 grams force on the footpad. (No vocalization and/or foot withdrawal after five trials was defined as complete nociceptive block - 100% of maximum possible effect [MPE]). The duration of nerve block was calculated as the time that nerve block was > 50% MPE. The test was applied every 30 minutes for the first hour and then hourly thereafter until full recovery. Laser irradiation (808 nm CW NIR laser) at 75, 141 and 272 mW/cm^2 was conducted for 10 minutes on both feet from day 2 to 5 after injection. The area under the curve (AUC) of the %MPE-time plot was computed using the trapezoidal method.[11] Fluorescence intensity of R6G in the injection spot was detected using an *in vivo* imaging system (IVIS spectrum, Caliper Life Sciences) with excitation and emission wavelengths of 535 nm and 580 nm before and after laser irradiation immediately. The local anesthetic effect of Lip-TD and Lip-GNR-0 and the effect of laser irradiation were studied. 100 µL of formulation was injected into the left footpad and the local anesthesia was assessed by the same procedure. All rats were irradiated at the injection site 24 hours after injection with an 808 nm CW NIR laser (141 mW/cm^2, 10 minutes).

Histology. All rats were euthanized with carbon dioxide 8 days after injection. All treated rat footpads were dissected and processed for histology.

Statistics. Data which were normally distributed were described with means and standard deviations. Otherwise, data were presented as median ± quartiles. The AUC of the %MPE-time curve for the plot of triggered local anesthesia at different laser irradiances was compared by two-way ANOVA (GraphPad Prism 5.0 software, GraphPad).

Study approval. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 150 to 200 grams were cared for in accordance with protocols approved by the Animal Care and Use Committee at Children's Hospital, and the Guide for the Care and Use of Laboratory Animals of the U.S. National Research Council.[12] They were housed in groups, in a 7 am to 7 pm light-dark cycle.

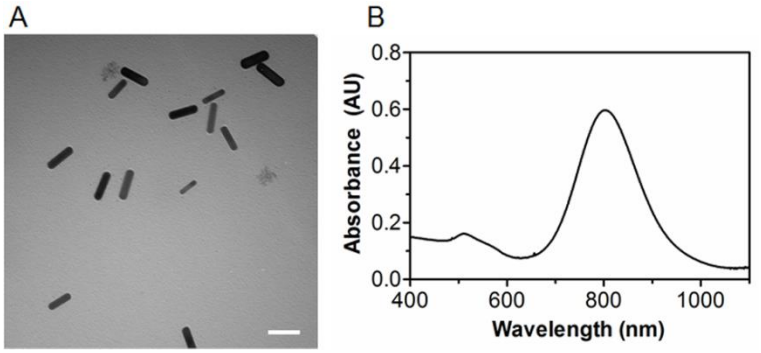

Figure S1. Characterization of gold nanorods (GNRs). Transmission electron microscopy image (scale bar 50 nm) and UV-visible spectrum of GNRs suspended in water.

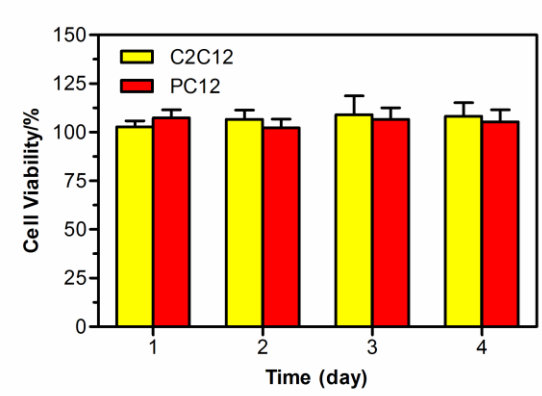

Figure S2. *In vitro* cytotoxicity assays. C2C12 and PC12 cells were cultured with TTX/DMED loaded liposomes conjugated with gold nanorods (Lip-GNR-TD) for 1-4 days (see Methods). Cell viability was assessed by the MTS assay. Data are means ± SD (n = 4).

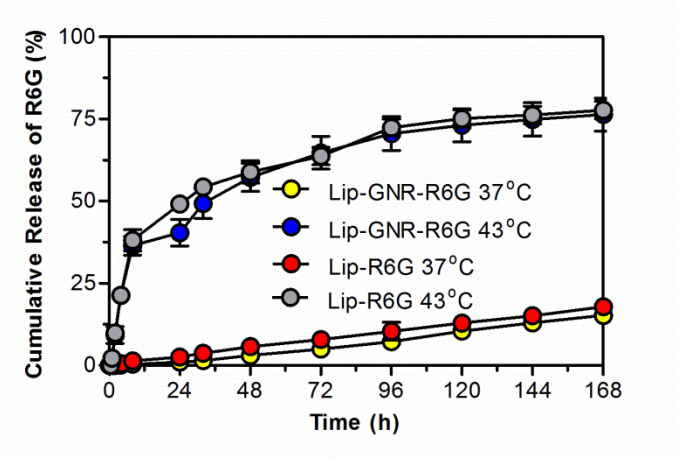

Figure S3. Effect of temperature on cumulative release of R6G from liposomes with (Lip-GNR-R6G) and without (Lip-R6G) gold nanorods. Data are means ± SD (n = 4).

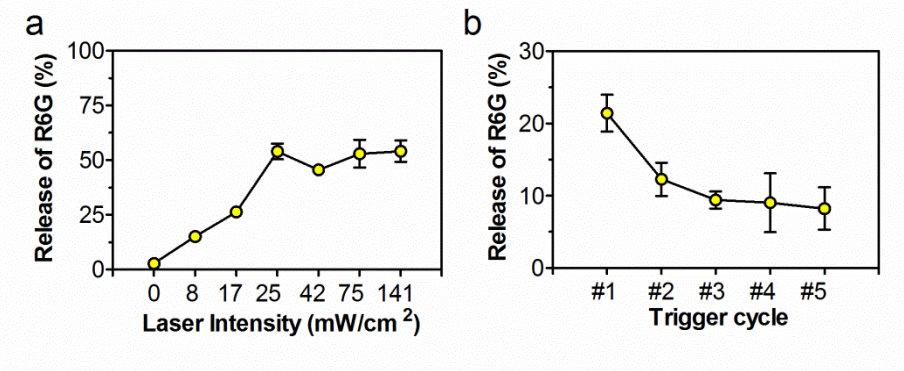

Figure S4. Phototriggered release of R6G by an 808 nm continuous wave NIR laser. (a) Effect of laser irradiance on R6G release from liposomes conjugated with gold nanorods (Lip-GNR-R6G). Irradiation lasted 10 minutes. (b) Repeated release of R6G from Lip-GNR-R6G from multiple 10-minute irradiations at 17 mW/cm^2. Data are means ± SD (n = 4).

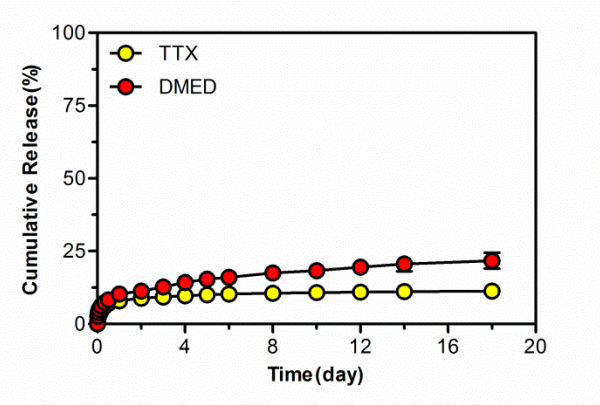

Figure S5. Release kinetics of TTX and DMED from TTX/DMED loaded liposomes conjugated with gold nanorods (Lip-GNR-TD) at 37 ^{o}C. Data are means ± SD (n = 4).

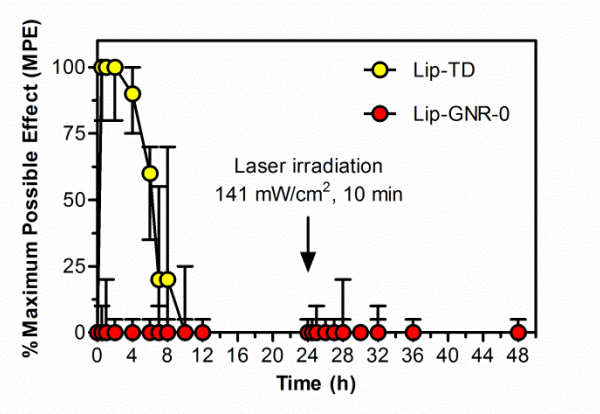

Figure S6. Effect on local anesthesia of the footpad (% maximum possible effect; see Methods) of injection of TTX/DMED loaded liposomes (Lip-TD, red circles) and blank liposomes conjugated with gold nanorods (Lip-GNR-0, blue circles). A 10-minute laser irradiation (black arrow, 808 nm continuous wave NIR laser) at 141 mW/cm^2 was conducted at the injection site of all rats 24 hours after injection and did not induce nerve blockade. Data are medians with 25th and 75th percentiles (n = 6).

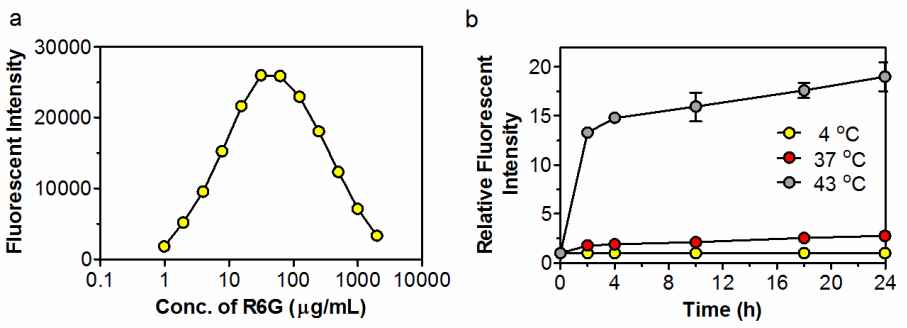

Figure S7. Self-quenching effect of rhodamine 6g (R6G). a) Effect of concentration of R6G in PBS on the fluorescence intensity, showing the self-quenching effect of R6G when the concentration is higher than 31.25 μg/mL. b) Relative fluorescent intensity of a solution of Lip-GNR-R6G after incubation at 4, 37 and 43 ^{o}C (see Methods). Fluorescent intensity was measured at RT and normalized to that before incubation. Data are means ± SD (n = 4).

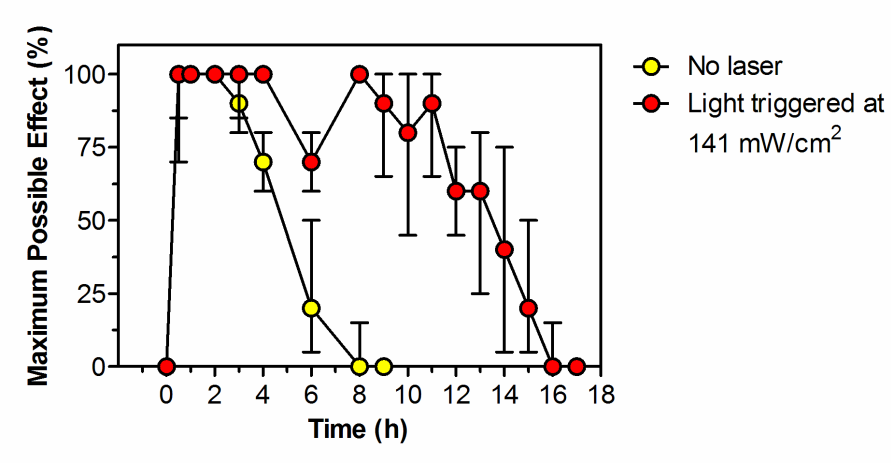

Figure S8. Prolonged duration local anesthesia after four laser irradiations. Local anesthesia is presented as % maximum possible effect; see Methods. Data are medians with 25th and 75th percentiles (n = 4). In the light triggered group, rats were triggered with 808 nm continuous wave NIR laser (141 mW/cm^2, 10 minutes) four times. Each triggering was conducted when the % MPE was less than 100%. Individual irradiation events are not shown since their timing varied from rat to rat.

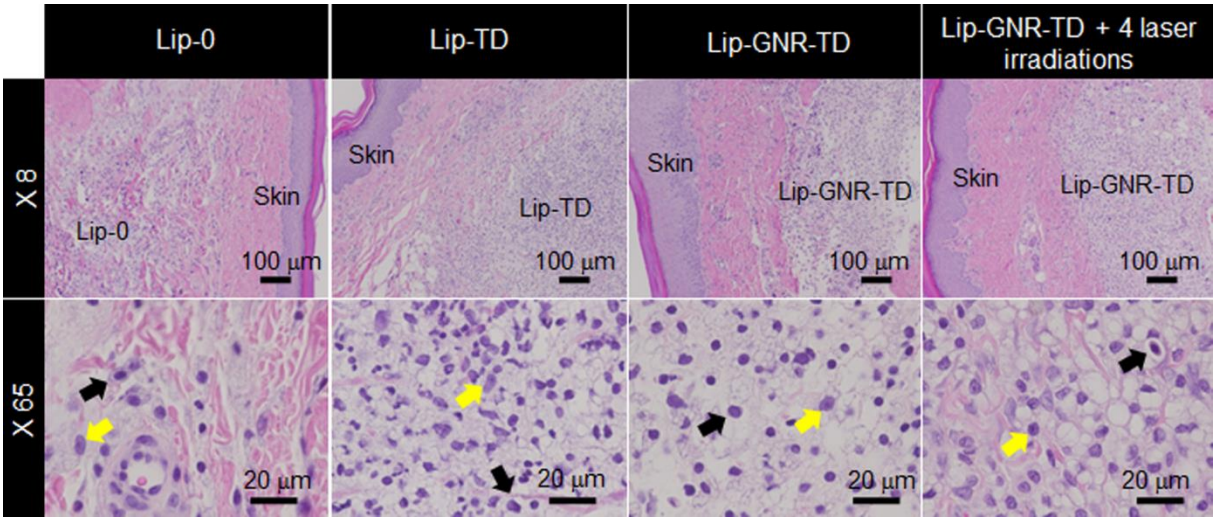

Figure S9. Representative light microscopy of hematoxylin/eosin-stained sections of rat footpads 8 days after treatment with Lip-0, Lip-TD, Lip-GNR-TD (all with no irradiation), and Lip-GNR-TD + four cycles of laser irradiation (141 mW/cm^2, 10 minutes). There was no damage to the skin (Skin) and underlying tissues. Inflammation at the injection site was characterized by macrophages (yellow arrow) and lymphocytes (black arrow) in all groups.

Table S1. Characterization of formulations. Data are means ± SD (n = 4).

Formulation	Concentration (mg/mL)			
	TTX	DMED	R6G	Lipid
Lip-GNR-R6G	--	--	2.33 ± 0.19	64.6 ± 2.21
Lip-GNR-TD	0.21 ± 0.02	0.17 ± 0.02	--	63.3 ± 3.23
Lip-R6G	--	--	2.04 ± 0.23	64.2 ± 2.54
Lip-TD	0.22 ± 0.03	0.15 ± 0.04	--	65.3 ± 5.08

Table S2. The duration (h) of local anesthesia in the left rat footpad after injection of TTX/DMED loaded liposomes conjugated with gold nanorods (Lip-GNR-TD). Data are medians with 25th and 75th percentiles (n = 4−6 per group; for the initial local anesthesia, n = 14 for the 3 groups).

Time of injection (h)	Blockade duration (h)					
	75 mW/cm^2		141 mW/cm^2		272 mW/cm^2	
	Left foot	Right foot	Left foot	Right foot	Left foot	Right foot
0 (Injection, no trigger)	5.0 (4.0-7.2)	0 (0-0)	5.0 (4.0-7.2)	0 (0-0)	5.0 (4.0-7.2)	0 (0-0)
24 (trigger #1)	0.5 (0.1-0.9)	0 (0-0)	1.5 (1.0-2.2)	0 (0-0)	3.0 (2.0-5.5)	0 (0-0)
48 (trigger #2)	0.5 (0.1-0.9)	0 (0-0)	1.0 (0.8-2.0)	0 (0-0)	2.5 (1.2-3.8)	0 (0-0)
72 (trigger #3)	0.5 (0.1-0.9)	0 (0-0)	1.0 (0.8-2.0)	0 (0-0)	1.5 (1.0-2.0)	0 (0-0)
96 (trigger #4)	0.5 (0.5-0.9)	0 (0-0)	0.8 (0.4-1.0)	0 (0-0)	0.8 (0-1.8)	0 (0-0)

Reference

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