Molecular machines open cell membranes

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An important need for future biomedical therapy is the effective delivery of drugs and genetic information into cells. Beyond the more common chemical delivery strategies, several physical techniques are used to open lipid bilayers of cellular membranes. These techniques include electric and magnetic fields, temperature, ultrasound and light, to introduce compounds into cells, release molecular species from cells, or to selectively induce apoptosis or necrosis. It was not until recently that scientists started exploiting molecular motors and switches that can change their conformations in a controlled manner upon external stimuli, to produce useful work for biomedical applications. Here we use molecular mechanical action to open cellular membranes by association of molecular motors with lipid bilayers, and then activating the motors with light. Using precisely designed molecular motors and complementary experimental protocols, molecular mechanical action can (a) induce the diffusion of analytes out of synthetic vesicles, (b) enhance diffusion of traceable molecular machines into and within live cells, (c) induce necrosis, (d) introduce analytes into live cells, and (e) be selectively targeted to specific live cell-surface recognition sites through nanomachines bearing short peptide addends. It is highly unlikely that a cell could develop a resistance to molecular mechanical action; therefore, beyond in vitro applications demonstrated here, in vivo use might follow, especially as molecular machine designs progress into the two-photon-, near-infrared- or radio-frequency-activated domains.

A scheme for molecular mechanical action upon a lipid bilayer is shown in Fig 1a and the general design of a molecular machine suitable for transport though a lipid bilayer is shown in Fig. 1b. The general features of a nanomachine are a rotor, bound to a larger stator, and
functional addends that can be appended to the stator for required solubility, tracking or cell surface recognition. As seen in Fig 1c. These include molecular motors bearing fluorophores for tracking (1 and 2), smaller nanomachines (3 and 4), a control that bears a stator but no rotor (5), a control analogue (6) that can only undergo cis-trans isomerization (flapping) at room temperature, and targeting systems that bear peptide sequences for binding to specific cell-surface receptors (7-10). We previously demonstrated that molecular machine 1 displays enhanced diffusion in solution when the fast light-driven motor is activated by 355 to 365 nm UV light. A fast unidirectional rotating motor in the 2 to 3 MHz range is essential for the expedited motion of the molecular-sized entities when overcoming the effects of Brownian motion, and there was no induced motion on a series of control molecules. We conjecture that similar motor-bearing nanomachines could be activated while associated with lipid membranes, and that is studied here.

As a first step to investigate molecular mechanical action, synthetic bilipid vesicles were opened using nanomachine 1 to release BODIPY dyes that were co-encapsulated with 1 in the vesicle (Extended Data Fig. 2). The release of BODIPY dye molecules (not nanomachine-bound) encapsulated in a bilipid vesicle was studied with UV-exposure, and there was little release of the BODIPY dye from the synthetic vesicle. Next, BODIPY and 1 were co-encapsulated in the bilipid vesicles, and a UV light-emitting diode was used as the activation source for 1 (Extended Data Fig. 3). As the UV irradiation time increased, the fluorescence intensity of the vesicles declined as BODIPY and 1 diffused out of the vesicles, suggesting molecular mechanical disruption of the vesicle bilipid membranes. A series of control molecules were used to exclude the possibility that the large fluorescence intensity drop in the vesicles containing the mixture of BODIPY and 1 was caused by the UV light induced photo-bleaching. The thermal effects due to
the absorption of UV light by 1 was not responsible for the vesicle opening since a control molecule that has an even larger absorption coefficient at 365 nm than that of 1 did not show the loss of BODIPY fluorescence from the vesicles (Extended Data Fig. 3).

Following the synthetic bilipid vesicle experiments, molecular mechanical action upon live cells was studied using confocal microscopy aided by a super-resolution technique called Phase Modulation Nanoscopy (see Extended Data for a complete overview of the equipment)\textsuperscript{16,17}. All live cell imaging experiments used either NIH 3T3 mouse skin fibroblast cells, PC-3 cells, a grade 3 human prostate adenocarcinoma, or Chinese hamster ovary cells (CHO). These are all well-studied cell lines with discrete morphological compositions. Two experimental methods were used: (Method A) the molecular motors were loaded into the cell media and the imaging was initiated within 5 min to 24 h, or (Method B) the molecular motors were loaded into the cell media, incubated for 30 min to 24 h, then washed three times with fresh molecular motor-free media before imaging. Each molecular machine was used as a stock solution at 0.10 to 1.00 $\mu$M with total dimethyl sulfoxide (DMSO) concentrations not exceeding 0.1% in the final cell media in order to avoid unwanted cell membrane permeabilization; the DMSO being required for solubility of the organic nanomachines (see Methods for more details).

The effect of the nanomachines on the cells was first studied without UV-light exposure. Using Method A, molecular machines 1 ($\lambda_{ex}$ 633 nm, $\lambda_{em}$ 650 to 680 nm for the pendant cy5 dyes) and 2 ($\lambda_{ex}$ 514 nm, $\lambda_{em}$ 530 to 580 nm for the pendant BODIPY dyes) do not induce accelerated necrosis when introduced to NIH 3T3 cells. However, due to 1 and 2 having well-pronounced traceable visible fluorescence properties corresponding to the cy5 and BODIPY addends (Extended Data Fig. 4 and Extended Data Table 1), respectively, their intracellular uptake, motion or protein/organelle-assisted trafficking are clearly observed. The two
luminescent compounds display very different localization patterns. Nanomachine 2 enters the cell and localizes in the mitochondria (Fig. 2a). Conversely, 1, when introduced to cells, displays pit-like cell surface localization (Fig. 2b and time-lapsed videos, Extended Data Videos 1, 2) and later, at 4 h, small ~1 μm aggregates are seen inside the cytoplasm. These respective time-dependent localization patterns were observed to be constant within the applied 0.10 to 1.00 μM final nanomachine loading concentrations, thereby suggestive of an active uptake mechanism. In order to confirm active molecular motor uptake and suspected endocytosis, a series of control experiments were undertaken using a range of nanomachine loading concentrations (0.10 to 1.00 μM, Method A) and incubation times of 5 to 60 min at 4 °C which is a temperature of general endocytosis inhibition. These studies reveal that there was no detectable localized fluorescence of 2 in the mitochondria or 1 on the cell surface. These experiments eliminate the possibility of passive concentration gradient-driven diffusion or static cell surface interactions of these nanomachines. Further strengthening this observation are the fluorescence intensity measurements where >99% of motors applied could be recycled from the wash solutions and re-collected during loading and imaging. Note that all loading experiments are carried out in a light-suppressed manner and the possibility of induced or accelerated uptake due to interaction between the molecular motors and the applied co-stain has been eliminated using a series of individual and reversed loading experiments. Since the motors were not UV-activated, there is no noticeable accelerated cell death. Cell viability throughout these experiments remained at >90% and both fluorescent nanomachines 1 and 2 were found to be non-toxic in the applied time and concentration regimes.

The NIH 3T3 cells in the presence of the nanomachines were then studied with concomitant UV activation. Upon UV-induced motor activation for 150 s (355 nm), 1,
introduced by Method A, was found to cross the cell membrane, and it was internalized into cells in a time-dependent manner, displaying fast accelerated intracellular motion (Extended Data Videos 3, 4 and 5), compared to natural homeostatic cellular organelle movement in the absence of UV-molecular mechanical activation (Fig. 2c, Extended Data Video 6). Combined controlled time and UV-exposure-dependent experiments indicate that the small aggregates of 1 inside the cytoplasm dissolve or burst with further increasing of fluorescence signal in the cytoplasm (Fig. 2d, comparing Extended Data Videos 3, 4 and 5 with Extended Data Videos 7 and 8). Thus, nanomachine trafficking can be facilitated and precisely observed.

We then proceeded to use the smaller nanomachine, 3, which, though difficult to track due its lack of fluorescent addends, proved to be the most active in molecular mechanical action, possibly due to its smaller size and ability to interact well with the cell membranes. Also, being addend-unencumbered, the rotor portion could easily be in close proximity to or within the membranes. Initial control experiments (blank) were performed without nanomachines being present. UV-induced (355 nm) PC-3 necrotic cell death is not initiated until approximately scan 20 (corresponding to 300 s continuous scanning UV exposure, 400 nJ/voxel total dwell time), characterized by massive disruption and rupture of the mitochondrial network and subsequent well-pronounced auto-fluorescent signal detectable in the nucleoli and nucleus wall (Fig. 3a). This is observable by the nucleoli membrane permeabilization and DNA damage at the onset of cell necrosis. The UV-induced necrotic cell death reaches final stages at approximately scan 40 (corresponding to 600 s) that is consistent with characteristic extracellular membrane rupture and homogenous cytosolic auto-fluorescence, indicating loss of cellular organelle boundaries. Conversely, visual signs of apoptosis involve cell shriveling and subsequent detachment from the cover-slip surface followed by fragmentation. Using 3 by Method A with both PC-3 and NIH
3T3 cell lines in the time- and UV-exposure-dependent *in vitro* microscopy experiments with the previously established standard experimental parameters and 355 nm laser exposure, >50% accelerated cell death (relative to UV-exposure without 3) is observed due to disruption of the cell membrane (Fig. 3b and Extended Data Fig. 5). Well-pronounced fragmentation of the mitochondrial network is established between scan 8 and 10 (corresponding to 120 to 150 s, respectively) with extracellular membrane burst manifesting at scan 20 (300 s) (Extended Data Table 2 and Extended Data Fig 5). This is confirmation of accelerated necrotic cell death. Importantly, 3 was found to be non-internalizing prior to UV-activation; onset of necrosis and internal cell organelle mitochondrial fragmentation or nucleolus damage is identical regardless of prior 2 to 4 h incubation of the cells with 3 vs. adding 3 directly before UV-activation.

Molecular machine 4 was studied using identical pre-set study parameters and Method A on both PC-3 and NIH 3T3 cells, where UV-induced molecular mechanical action caused necrosis only 10% earlier (Extended Data Table 2) than the standard blank non-molecular motor containing reference cells (Fig. 3c and Extended Data Fig. 5). Nanomachine 4, bearing the larger aryl sulfonate moieties might have been inhibited from having its rotor interact well with the cell membranes, or the addends themselves sterically encumbered their rotation near the membrane.

The rotor-free control molecule 5 was studied to ensure that the rotary action is essential for the bilayer perturbations. Using Method A on both PC3 and NIH 3T3 cells, which has the same homopropargylic alcohol stator moieties as does 3, control molecule 5 shows no effect on cell necrosis upon standardized UV-exposure (Fig. 3d, Extended Data Fig. 5). This further suggests that the accelerated cell death seen with 3 was not primarily due to the short exposure to UV light or subsequent thermal processes, as similarly attested by the above-noted work with the synthetic bilayers.
In order to further ensure that a fast rotary motion was essential for molecular mechanical opening of cells, another control (6) was used which bears a 6-membered heterocyclic rotor that is nearly identical in molecular size and functionality to 3. However, 6 can only undergo cis-trans isomerization upon light activation at room temperature. This “flapping” action will occur but without any full rotation since that barrier (rotor crossing over the stator) requires 60 °C in the heterocyclic system. Even at that elevated temperature, the rotation rate is only ~2 revolutions·h⁻¹ as opposed to the nearly identical molecular sized 3 which rotates at 2 to 3 MHz upon UV-activation at room temperature. Compound 6 showed no enhanced necrosis in PC-3 or NIH 3T3 cells upon standardized UV-exposure (Extended Data Fig. 6). This further underscores the necessity of fast rotary action as opposed to the simpler cis-trans UV-light induced isomerization (flapping).

In order to further confirm the molecular mechanical opening and subsequent permeabilization of the membrane, a dye was added to the cell medium to assess its exogenous entry into the cells that might be afforded by molecular mechanical action. Using PC-3 cells and 3, propidium iodide (PI, total concentration 0.10 μM) was introduced to the cell medium immediately prior to the time-dependent standardized imaging sequence. PI is a fluorescent intercalating agent that is not internalized by healthy cells, and it is non-toxic as shown by our molecular machine-free controls. Upon membrane disruption by molecular mechanical action of 3 (Fig. 3e), PI enters the cell, travels to RNA- and DNA-rich areas where it intercalates and its excitation maximum subsequently displays ~30 nm bathochromic shift (from 535 to 565 nm) accompanied by a parallel hypsochromic emission maximum shift (from 617 nm to 600 nm), consistent with literature-noted trends. Although PI’s molar extinction coefficient is relatively low compared to other organic cell dyes, upon intercalation, its red fluorescence emission
intensity increases by >10x, thereby becoming a suitable counter-stain to assess and establish the onset of UV-induced necrosis. In light of its favorable spectroscopic properties, no major alteration of the experimental parameters are needed other than exchange of the 488 nm laser line, used to generate transmission images, to 543 nm (0.2 mW, also confirmed to be non-disruptive to cells). Internalized RNA- and DNA-induced PI fluorescence is detected between 600 and 630 nm allowing time-dependent light-activated molecular motor-induced cell permeabilization to be confirmed. Further, PI was used to follow membrane damage that is due to UV-activated nanomachine activity leading to necrotic cell death. Since the entry of the PI is on a relatively short time scale compared to the time of cell division, the cell has insufficient time to adopt programmed cell death (apoptosis). This has been confirmed using Annexin V, an apoptosis-specific stain where no relevant fluorescence from this dye has been observed throughout the course of the experiments.

Considering the above UV-induced molecular mechanical action, the peptide-bearing structures (7-10) were investigated to target specific cells for nanomachine-activated necrosis. The targeted cell line was PC-3 while NIH 3T3 and sometimes CHO cells were used as non-targeted controls. The effect of 7 and 8 on PC-3 and NIH 3T3, and 9 and 10 on PC-3, NIH 3T3 and CHO cells was studied in their non-UV-activated states and noted as background controls. Whether using Methods A or B, accelerated cell death of the PC-3 cells was observed with both 7 and 8 from the onset of motor activation, although the necrosis manifested more slowly (20 to 25 % corresponding to 240 s) than when the smaller molecular motor 3 was used (Extended Data Table 2, Extended Data Fig. 7). Surprisingly, motors 7 and 8 also showed a strong association with NIH 3T3 cells where UV-activated molecular mechanical-accelerated necrosis occurred in the same timeframe as with the PC-3 cells, indicating no desired selectivity of PC-3 over NIH
3T3 cells (Extended Data Table 2, Extended Data Fig. 8).

Since no selectivity was observed with the shorter peptide targeting moieties, the longer peptide sequences provided by 9 and 10 were synthesized to see if selective targeting could occur on the PC-3 cells over the other two cell lines. Using Methods A and B for 9 over several concentrations (0.10 to 1.00 μM) and incubation times (5 min to 24 h), PC-3 cells started to die of UV-activated motor-induced necrosis at 150 to 180 s, which corresponds to 40 to 50% faster onset than the molecular-motor-free UV-exposed cells which took 300 s to show the same level of necrosis (Extended Data Table 2, Fig. 4a-c, Extended Data Fig. 9). Introduction of 10 to the culture media under identical experimental conditions shows a similar trend: 25 to 30% increase in necrosis observed at 210 s (Fig. 4d-e, Extended Data Fig. 9). Both UV-activated 9 and 10 trigger necrosis faster than the UV-exposed motor-free PC-3 cells. The observations of both PC-3 and NIH 3T3 cells incubated for 24 h are shown in Extended Data Fig. 9 and summarized in Extended Data Table 2. More notably, selective targeting of PC-3 over NIH 3T3 and CHO cells was observed with 9. With the introduction of 9 at 0.50 or 1.00 μM to NIH 3T3 or CHO cells and incubation for 1 to 4 h with no subsequent washing (Method A), no observed acceleration (<10%) of UV-induced cell death was observed (Extended Data Fig. 10 and 11 and Extended Data Table 2). Presumably in the case of 9 with the peptide addends at both ends of the stator, it cannot sufficiently bind to the cell membrane of the untargeted NIH 3T3 or CHO cells to induce significantly accelerated necrosis. Thus, pre-binding to the cell membrane is essential; just being present in the medium will not result in accelerated rotor-induced UV activated necrosis. Note that with 3, both NIH 3T3 and PC-3 cell showed accelerated activated necrosis presumably because the stator on 3, with its smaller addends, interacted well with the membranes, or it had minimal pendant interference while interacting with the membranes. In the case of 10 using
Method A, NIH 3T3 or CHO cells with UV-activate-nanomachine cell necrosis is established to 20% faster (up to 240 s continuous scan) than with 9 (Extended Data Fig. 10 and 11 and Extended Data Table 2). This suggest that the mono-addended 10 is better able to approach the membrane to sufficiently close proximity, albeit untargeted, than the more sterically hindered 9, but still not as efficiently as the smaller 3. Once necrosis manifests via activated 10, it accelerates the final necrotic stage by 20 to 30% over that of 9. This further suggests better transport through the membrane by the less sterically encumbered 10 over 9; pulling through the larger addends slows the membrane-transport process. When 9 or 10 at 0.50 or 1.00 μM are introduced to either NIH 3T3 or CHO cells, incubated 2 h, washed three times, and then imaged in rotor-free culture media (Method B), there was little difference in time of UV-activated necrosis (up to 10% with 9) to the initial control cell experiments. Motors 9 and 10 did not accelerate the necrosis on these cells (Extended Data Figs. 10 and 11) suggesting that the sequence SNTRVAP does indeed afford the desired selectivity towards PC-3 cancer cell GRP78 site over that of NIH 3T3 or CHO cells.

The dynamic effects of molecular mechanical action upon cellular membranes were then studied through the whole cell patch clamp electrophysiology of human embryonic kidney 293 cells (HEK293) commonly used for electrophysiological interrogation. Using Method B, the studies reveal that upon UV (355 nm) activation of molecular motor 3, inward ionic currents were produced consistent with hydrophilic pores forming in the cellular membranes. These inward currents were not observed in the absence of UV illumination or during UV illumination of non-rotor-bearing control 5 or UV illumination of untreated cells (Fig. 5a). Inward currents produced during UV illumination of cells treated with 3 then continued even in the absence of UV illumination suggesting that the cell membranes were irreversibly damaged. This was
accompanied by induced morphological changes to the cells, such as membrane blebbing (Fig. 5b, white arrows), cell swelling and cytoplasmic degradation, all indicative of cell death. Although membrane blebbing occurs during apoptosis and necrosis, the large diameter of the blebs observed here (Fig. 5b; \( r = 3.8 \pm 0.2 \) \( \mu m \)) matches necrosis as does the observed cell-swelling and the absence of apoptotic bodies. Consistent with our previously observed delayed morphological effects on the other cell lines studied above, inward currents in HEK293 cells appeared between 40 and 60 s after exposure to UV illumination. The slow rise in inward current during illumination suggests an accumulation of many small pores or increasing pore sizes.

An evaluation of the forces produced by the UV-activated molecular machines upon the bilipid membranes is considered here. Membrane rupture and pore formation under a tangential mechanical force have been studied theoretically and experimentally. Generally, when the stress of the membrane exceeds their critical value, membrane rupture occurs. The critical rupture tension of biological membranes varies from 1 to 30 mN m\(^{-1}\), depending on the specific chemical composition of the membrane. It has also been shown that the rupture stress is dependent on the stress loading rates. For example, Li et al. showed that in impulse stretching experiments over tens of \( \mu s \), red blood cell membranes can sustain the stress by >10x over stresses induced by quasi-static stretching conditions. Further, pore formation is a highly dynamic process whereupon the pores either close or continue to grow until rupture of the membrane. Under tension, pore formation becomes faster, though the pore formation is a transient event which is challenging to capture.

Specific to the molecular mechanical forces in our experiments, the actuation of the rotor will produce a tangential mechanical force perturbing the membrane structure. The UV
photon energy ($\lambda = 365$ nm) that actuates the motor is $E = \frac{hc}{\lambda} = 5.4 \times 10^{-19}$ J. If the entire amount of energy is used for the force generation, and the linear moving distance of the tip of the rotor is on the magnitude of $s = 1$ nm, the generated force would be $F = \frac{E}{s} = 0.54$ nN. The stress applied on the membrane would be 540 mN m$^{-1}$, far exceeding the requisite rupture stress for most bilipid membranes of 1 to 30 mN m$^{-1}$ (ref 25, 27, 31). Even if we consider that the molecular mechanical action is pulsed and the membrane is more resistant to rupture, it is still theoretically sufficient to disrupt the membrane locally and to eventually compromise its integrity. This conclusion is also consistent with the energetics estimation. The estimated free energy for pore formation is tens of kJ mol$^{-1}$ using molecular dynamics simulations$^{34, 35}$. The corresponding UV photon energy is sufficient to disrupt ~10 lipid molecules to form a transient pore. Further, the disruption effect of motor actuations might be cumulative. Considering that the rotors (~1 nm) are small relative to the thickness (7.5 to 10 nm) of the bilipid membranes, the rupture kinetics of the observed molecular mechanical opening is not expected to be immediate. This is consistent with our experiments here as well as the delayed membrane openings seen by others using probe-induced mechanical perturbations$^{36, 37}$, accepting, however, that probe-tip perturbations are a vertical force model and hence considerably different than the tangential molecular mechanical effects. Therefore, molecular mechanical action can generate a concerted motion upon a 1-nm-long molecular rotor that will severely dislocate the membrane molecules, while other light absorbing molecules will merely dissipate the absorbed energy in random motions of atoms in the molecule, underscoring the efficacy of the molecular mechanical effect for membrane disruption.

Based upon the studies performed, the following features appear essential for molecular machine opening of cellular membranes. A rapidly spinning rotor is necessary; without rotation
there is no membrane perturbation. All of the nanomachines studied here have rotors that can rotate in 2 to 3 MHz regime\textsuperscript{38}, and there was sufficient rotary actuation for disruption (See Extended Data for further comments on motor rotation vs. photon flux). We do not know the lower limit for rotary efficacy, however. Smaller and addend-unencumbered molecular machines are preferred over the more encumbered systems. The molecular machines must embed in the membranes to show opening; merely their presence in the medium is insufficient. When targeting, it is preferred to have a targeting addend that does not impede rotor operation. If using two relatively large addends, they might retard the rotor from interacting with the lipid bilayer thereby slowing the molecular mechanical perturbation of the membrane. Since these are molecular-sized, pore formation on the membranes is not immediate; the process can take \(\sim 1\) min to become detectable through leakage currents and twice that long based upon morphological changes. Sufficient rupture stress will have to be displayed by the rotors in order to be effective in bilayer disruption. Finally, shorter UV-actuation times of \(<30\) s can permit analytes in the medium to enter the cells before the cells can reach the stage of programmed cell death.

In summary, molecular mechanical action can disrupt external or internal cellular membranes and it can be used to introduce analytes into cells or expedite cell death. By synthetic design, the nanomachines can be tracked within a cell or used to target specific cells through unique cell-surface recognition elements. The efficacy of this method for \textit{in vitro} studies was demonstrated. Extensions to \textit{in vivo} applications can be envisioned at locations where short UV-exposure is acceptable such is in dentistry, localized epidermis and colorectal treatments\textsuperscript{39}. Future molecular motors that might be activated by, for example, two-photon-, near-infrared- or radio-frequency-inputs, would make broader \textit{in vivo} treatments viable.
Methods

Toxicity measurements. Cell toxicity was determined using a ChemoMetec A/S NucleoCounter3000-Flexicyte instrument with Via1-cassette cell viability cartridge using the cell stain Acridine Orange for cell detection, and the nucleic acid stain DAPI for detecting non-viable cells and Annexin V for the detection of apoptosis. In cellular uptake studies, cells were seeded in 6-well plates and allowed to grow to 80% to 100% confluence at 37 ºC in 5% CO₂. Culture medium was then replaced with culture medium containing 0.1% DMSO with individual nanomachines 1-10 for 24 h at 0.10, 0.50 and 1.00 μM. All cell colonies bearing nanomachines displayed 92±5% viability; the control blank cells were established at 95±3% viability. In addition to 0.1% DMSO being used for molecular machine introduction, all washing solutions also contained 0.1% DMSO. At this concentration DMSO does not affect the cells; this was determined by control experiments using all imaged cell lines cultured in DMSO-free and 0.1% DMSO-containing cell media while establishing the initial control UV-induced cell death parameters. To confirm the non-activated low toxicity of these molecular machines, all live cell imaging samples with all three studied cell lines were re-incubated, using Method A, in the dark and re-imaged using only transmission microscopy using a tungsten bulb with a LP 420 nm cut off filter. These experiments confirmed that all previously non-UV-exposed cells, regardless of the cell line studied, still proliferated in the presence of the molecular motor stock solutions for up to 72 h using visible light at a pre-set time point to assess cell morphological changes along with viability and vitality.

Live cell microscopy parameters. All experimental imaging parameters (i.e., laser beam size, confocal pinhole size, laser intensity, line scanning speed, scanning area (field of view,
FOV), and line averaging sequences) were kept constant throughout the experiments. The accuracy and errors associated with the establishment of accelerated necrosis are determined based on one dual channel imaging sequence, which takes 15 s in total. This imaging sequence has been carefully established using untreated live cells. The optimized imaging parameters allow appropriately high scanning speed to follow natural homeostatic events and identify any induced morphological or fluorescent signal localization change. Meanwhile, they also allow sufficiently long integration time for each pixel so an adequate amount of photon signals can be collected. In order to satisfy the Nyquist sampling criteria, the pixel size is set as 1/5 of the laser spot size. The images were acquired using a bidirectional 2-line averaging sequence, which gives minimal dead time (<1 ms) between line scanning. The image size was adjusted to 100 × 100 μm in order to study 1 to 3 cells simultaneously. Each individual experiment was repeated 3 times on triplicate slides. On each slide, at least 5 well-separated areas were imaged. See the Extended Data explanations and Extended Data Table 2.

**Imaging of live cells.** Steady state fluorescence images were recorded using a PhMoNa enhanced Leica SP5 II LSCM equipped with a HCX PL APO 63x/1.40 NA LambdaBlue Oil immersion objective. See Extended Data for more details. As an initial control experiment to establish the UV-induced cell toxicity threshold, while mitigating voxel exposure and ensuring sufficient laser dwell time, an imaging sequence has been established to monitor cell morphological and physiological changes as a function of time. All experiments used cell-line-determined culture medium containing 0.1% DMSO. The applied 100 Hz detection sequence was based on a bidirectional dual-channel continuous scanning method where a minimalistic non-damaging visible laser light (458 nm, 0.2 mW) is used in conjunction with the above detailed UV exposure. This is set as a 2 line/scan accumulation parallel acquisition sequence that
is recorded as a function of time. Studied channels correspond to transmission images and UV-induced mitochondrial auto-fluorescence detected at 460 to 550 nm. We further confirmed that the effectiveness of monitoring the UV-activated molecular mechanical action on live cells using a conventional CW mercury-arc excitation source equipped with an epi-fluorescence setup consisting of a Zeiss Axivert 200M inverted microscope, as discussed in Extended Data Fig. 12.

**Electrophysiology.** HEK293 cells (CRL-1573 from ATCC) were cultured in DMEM supplemented with 10% FBS and 1% pen-strep. One hour prior to recording, cells were incubated in DMEM containing 1 μM of the test compound. The cells were placed in a recording chamber filled with extracellular recording buffer (in mM: 145 NaCl, 5 KCl, 3 MgCl₂, 10 HEPES and 1 CaCl₂; pH 7.2; adjusted to 320 mOsm with sucrose). Glass patch pipettes with a resistance of 3 to 5 MΩ were filled with intracellular buffer (in mM: 140 KCl, 10 HEPES and 0.5 EGTA; pH 7.2; adjusted to 320 mOsm with sucrose) and brought into contact with the cell membrane to generate seals ≥ 1 GΩ. A negative pressure of −70 mmHg was applied inside the pipettes to gain access to the whole cell configuration. An Axopatch 700A amplifier was used to monitor currents under voltage clamp conditions. The current was filtered at 10 kHz and digitized at 2 kHz using a Digidata 1550 (Molecular Device). The UV light (355 nm, 2.6 mW) was produced by an XLED1 from X-Cite and the timing of the illumination was controlled by the Axopatch. UV illumination and DIC imaging were performed using a Nikon TE-2000S inverted microscope and a 100 X, 0.9 NA objective lens.


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**Supplemental Information**

The synthetic details, NMR spectra, HPLC chromatograms, and further explanation of the optical studies on synthetic vesicles and live cells are described in the Extended Data.

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Author contribution

The idea to use the nanomachines to open membranes was suggested by J.M.T., then further discussed this with G.W. and R.P. V.G.L. carried out the synthesis of $1-13$ and $16$, and L.G.N. carried out the synthesis of $14$, both under the supervision of J.M.T. F.C. performed the experiments on lipid vesicles under the supervision of G.W. R.P. carried out all the experiments on live cells. A.A. and V.G.L. carried out the HPLC separation of $7-10$ in the laboratory of Professor Angel Martí. A.B.K. and G.W. developed the theory. G.D. performed the patch clamp work under the supervision of J.T.R. and they prepared the text for that section. V.G.L., G.W., R.P., and J.M.T. wrote the manuscript. All authors read and approved the manuscript.

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$R = \text{functional addends}$
Figure 1 | Molecular motors for disruption of lipid bilayers through molecular mechanical action and control molecules. (a) Schematic of a molecular machine approaching and then disrupting a cell membrane by UV-activated molecular mechanical action. (b) The representative molecular machine shows the rotor portions in red which are light-activated to rotate relative to the larger bottom blue stator portion; the green addends (R) can be varied to provide the requisite solubility, fluorophores for tracking, or recognition sites for cellular targeting. (c) Nanomachines 1 and 2 bear fluorophores as pendants on the stator portions for tracking their movement, and the visible absorbance of their cy5 and BODIPY fluorophores, respectively, are optically separated from the motor ultra-violet (UV) absorbance/activation. Molecular motors 3 and 4 have smaller molecular sizes than 1 and 2 but with no stator-addended fluorophores for their tracking. Compound 5 has a stator segment but no rotor; this serves as a control molecule that cannot be UV-activated. Nanomachine 6 has a slow rotor which serves as a control. Nanomachines 7 and 8 are functionalized with the DGEA peptide sequence to target α2β1-integrin overexpressed in PC-3 human prostate cancer cells; 9 and 10 are functionalized with SNTRVAP to bind to the 78 kDa glucose regulated protein (GRP78) targeting castrate-resistant osteogenic prostate cancer receptors on PC-3 human prostate cancer cells. Rotors in nanomachines 1-4 and 7-10 rotate at 2 to 3 MHz when activated with 355 to 365 nm light while 6 rotates at 1.8 revolutions per hour when activated with 355 to 365 nm light if at 60 ºC, but only cis-trans isomerizes about the rotor-stator double bond at room temperature. The syntheses are described in the Extended Data along with the photo-induced interconversion of the cis- and trans-isomers of 7 (Extended Data Fig. 1).
Figure 2 | NIH 3T3 cells in the presence of the fluorescent molecular machines 1 and 2 were studied with UV activation to cause molecular mechanical-induced entry of 1 and 2 into the cells. Laser scanning confocal microscope (LSCM) images of 1 and 2 in NIH 3T3 cells (1024 x 1024 pixel). (a) Nanomachine 2, left image (green, \(C_{\text{loading}}\) 500 nM/2 h, \(\lambda_{\text{ex}}\) 514 nm, \(\lambda_{\text{em}}\) 520-540 nm)
nm, 2 mW); middle image MitoTrackerRed (red, \( C_{\text{loading}} \) 100 nM/30 min, \( \lambda_{\text{ex}} \) 543 nm, \( \lambda_{\text{em}} \) 550-600 nm, 0.5 mW); right is the two merged transmission images verifying mitochondrial localization. (b) Nanomachine 1, left image (red, \( C_{\text{loading}} \) 500 nM/1 h, \( \lambda_{\text{ex}} \) 633 nm, \( \lambda_{\text{em}} \) 650-700 nm, 1 mW); middle image is LysoTrackerGreen (green, \( C_{\text{loading}} \) 200 nM/5 min, \( \lambda_{\text{ex}} \) 488 nm, \( \lambda_{\text{em}} \) 500-530 nm, 0.2 mW); right is the two merged transmission images highlighting pit-like surface localization. (c) Merged transmission (488 nm, 0.2 mW) images demonstration time dependent 1 internalization (red, \( C_{\text{loading}} \) 500 nM, \( \lambda_{\text{ex}} \) 633 nm, \( \lambda_{\text{em}} \) 650-700 nm, 1mW). UV-activation has been achieved using parallel \( \lambda_{\text{ex}} \) 355 nm, 20 mW 400 nJ/voxel total dwell time for the corresponding times noted in the images (See Extended Data Videos 4 and 5). (d) Fluorescent images demonstrating time-dependent dispersion of formed intracellular aggregates of 1 after 1 h incubation and wash cycles followed by UV-activation (red, \( C_{\text{loading}} \) 500 nM/1 h, \( \lambda_{\text{ex}} \) 633 nm, \( \lambda_{\text{em}} \) 650-700 nm, 1 mW) for the corresponding times noted in the images. All scale bars = 20 \( \mu \)m.
Figure 3 | The effects of nanomachines 3 and 4, and control molecule 5 on PC-3 cells upon UV-activation; the rate of necrotic cell death and permeabilization of analytes into the cells was recorded. Recorded merged transmission (458 nm, 0.2 mW) and UV-induced mitochondrial auto-fluorescence (green, $\lambda_{\text{ex}}$ 355 nm, $\lambda_{\text{em}}$ 460-550 nm, 20mW 400nJ/voxel total dwell time, 1024 x 1024 pixel) images of PC-3 human prostate cancer cells depicting time-dependent UV-activated molecular mechanical-induced cell morphological changes. The UV-exposure times are shown in each image. (a) Blank cells without molecular motors; (b) with introduction of 3, (c) introduction of 4; (d) with non-active 5 all at 500 nM with 5 min incubation before imaging. (e) Identical imaging sequence using 3 with the introduction of 100 nM PI (red, $\lambda_{\text{ex}}$ 543 nm, $\lambda_{\text{em}}$ 610-630 nm, 0.2 mW) confirming molecular mechanical cell permeabilization with intercalation of RNA and DNA primarily in the cell nuclei. All scale bars = 20 μm. The statistical analyses for each of the live cell microscopy experiments are shown to the right of the images for that row. The determination of onset (orange) and final stage (red) of necrosis are shown combining: 4 to 6 individual microscope slides with 5 to 6 FOV on each with an average 2.5 to 3.1 cells per FOV. The displayed standard deviations are calculated from the Gaussian fit and have been rounded up to 15 s integers due to the experimentally predefined length associated with each scanning sequence. See Extended Data Table 2 for more details.
Figure 4 | Study of targeted molecular mechanical action of 9 and 10 upon PC-3 cell necrosis. Recorded merged transmission (458 nm, 0.2 mW) and UV-induced mitochondrial auto-fluorescence (green, $\lambda_{ex}$ 355 nm, $\lambda_{em}$ 460-550 nm, 20mW 400nJ/voxel total dwell time, 1024 x 1024 pixel) images of PC-3 human prostate cancer cells depicting time-dependent UV-activated molecular mechanical-induced cell morphological changes. PI was added to all the cell media. The UV-exposure times are shown in each image. (a) Blank cells without molecular motors; (b) with introduction of 9 without washing; (c) with introduction of 9, followed by washing; (d) with introduction of 10 without washing; (e) introduction of 10 followed by washing. All scale bars = 20 $\mu$m. The statistical analyses for each of the live cell microscopy experiments are shown to the right of the images for that row. The determination of onset (orange) and final stage (red) of necrosis are shown combining: 3 to 6 individual microscope slides with 5 to 9 FOV on each with an average 2.5 to 3.1 cells per FOV. The displayed standard deviations are calculated from the Gaussian fit and have been rounded up to 15 s integers due to the experimentally predefined length associated with each scanning sequence. See Extended Data Table 2 for more details.
Figure 5 | Whole-cell patch clamp studies of the dynamic effects of UV-induced molecular mechanical action of 3 upon HEK293 cells. Also shown are control studies using 3 without UV activation, UV-exposed rotor-free control molecule 5, and no molecular additives x (n = 3 cells per condition). Compounds 3 and 5 were used at 1.0 μM concentrations. (a) Transmembrane currents in HEK293 cells show that cells treated with UV (355 nm)-activated molecular motors 3 have inward currents consistent with membrane degradation (bottom trace). Without UV illumination, cells treated with 3 or 5 show no change in membrane currents over the 4-min recording period (top two traces). Similarly, the rotor-free 5, and no molecular additive, x, show no inward currents during UV illumination (center two traces). Cells were held at −70 mV in voltage clamp mode and UV exposure began 15 s after the start of the recording. The gray shaded region represents standard error of the mean, and black traces represent the average of 3 experiments.
for each condition. (b) Representative differential interference chromatography (DIC) images of cells captured before (t = 0) and after (t = 4 min) exposure to UV in the presence of 3 or 5. The white arrows (lower right image) highlight membrane blebbing that appears only in the cells treated with UV-activated 3. The scale bar represents 10 μm and is applicable for each micrograph.
Molecular machines drill holes in cells