Published on Mar 17, 2025 by Arcadia Science **DOI**: 10.57844/arcadia-3207-4695

Compound 48/80 is toxic in HMC1.2 and RBL-2H3 cells

We found that compound 48/80, an MRGPRX2 agonist and commonly used *in vitro* mast cell activator, is toxic in HMC1.2 (human mast cells) and RBL-2H3 (rat basophils). Researchers should use caution and incorporate a viability test when performing assays in these cell lines.

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Version 1 · Mar 31, 2025

Purpose

We're interested in leveraging tick biology to develop new drugs for itch and inflammation. Toward this goal, we're developing *in vitro* assays to study these processes and to test potential interventions. Given the importance of mast cells in host detection of ticks and other skin ectoparasites, we decided to use a mast cell degranulation assay that detects release of β-hexosaminidase into the supernatant medium to test whether our test molecules inhibit this type of immune activation.

In the process of developing this assay, we learned that 48/80, one of the most commonly used mast cell activators, causes cell lysis at concentrations typically used to induce degranulation. Because this fact raises concerns about the interpretability of data generated using 48/80 and hasn't yet been widely reported in the literature, we're sharing it here.

- **Raw data** and associated **code** from this pub are available in this GitHub repo.
- Detailed **experimental protocols** are available as a collection on protocols.io.

We've put this effort on ice! ☒

> **#TechnicalGap**
>
> Because we haven't been able to identify an activator that 1) is relevant to the biology we're studying and 2) can induce degranulation in our model without causing significant toxicity, we're icing this assay as a strategy to test the putative tick anti-inflammatory molecules we identified. We're exploring alternative experimental strategies to test these molecules' activity, including qPCR.
>
> Learn more about the Icebox and the different reasons we ice projects.

Motivation

Mast cells are tissue-resident innate immune cells that sense infection and other disruptions to tissue homeostasis, particularly in the skin, and respond to these threats by inducing inflammation and activating other immune cells **[1]**. Although they diverge during their development from the common progenitor of circulating granulocytes **[2]**, mast cells also contain granules filled with inflammatory mediators, and they activate downstream effector responses by releasing these mediators in a process called degranulation **[1][3]**.

Mast cells are also known to play important roles in various signaling pathways that lead to itch **[3][4][5]**, an important host defense mechanism against ectoparasites. Because they serve this important sentinel function, ticks have evolved strategies to suppress mast cell activation in order to effectively evade host detection during their extended feeding periods **[6][7][8][9]**.

We've identified a set of molecules from ticks that we believe may play a role in their success as long-term blood feeders **[10]**. Although we don't know the targets of these tick molecules, we reasoned that mast cells are strong candidates because they are important for initiating itch and inflammation in the skin. We therefore decided to test whether these molecules could inhibit mast cell degranulation.

The approach

Don't need methodological details? Skip straight to the results.

We chose to perform an *in vitro* mast cell degranulation assay since it would offer improved throughput compared to *in vivo* experiments. To achieve this, we needed to select cell lines that we could use to model the degranulation process, identify activators that could be used to induce it, and develop an assay to detect it. Based on the literature in the field, multiple options are available for each of these objectives, but there weren't specific examples of every possible permutation of model, inducer, and readout.

> **TRY IT**: Our collection of protocols for mast cell culture, activation, and degranulation/viability analysis are available here (DOI: 10.17504/protocols.io.5jyl8dqr7g2w/v1).

Selection of HMC1.2 and RBL-2H3 cells

We explored two mast cell models for this assay. Researchers favor the HMC1.2 and RBL-2H3 cell lines as mast cell models due to their straightforward culture procedures and rapid doubling times **[11][12]**. HMC1.2 cells are a human mast cell leukemia line **[11]**, while RBL-2H3 cells are rat leukemia cells **[12]**. While the latter are typically

classified as basophils, they exhibit a hybrid phenotype, with some characteristics more closely resembling basophils and others more closely resembling mast cells **[12]**. RBL-2H3 cells have a number of crucial receptors in common with human mast cells, including IgE receptors **[12]** and Mrgprb2 (the rodent MRGPRX2 homolog) **[13]**. They're significantly more granulated than HMC1.2s **[14]**, making it easier to detect when they've been activated.

HMC1.2 cells are suspension cells that we grew in Iscove's modified Dulbecco's medium (IMDM) with 1.2 mM α-thioglycerol, 10% fetal bovine serum (FBS), and a pen-strep antibiotic cocktail. RBL-2H3 cells are adherent cells, which we cultured in minimum essential medium (MEM) with 15% FBS and pen-strep. We maintained both cell lines in sterile incubators with a 5% CO2 in air atmosphere. These are the maintenance conditions that EMD Millipore and ATCC, their respective sources, recommend.

Inducer preparation and treatment

While many inducers of mast cell degranulation are known, not all of these are relevant to our therapeutic discovery goals. For example, one of the most common approaches to induce degranulation in mast cells is IgE crosslinking, but this inducer is more relevant to allergic responses **[15]** and histaminergic itch **[16]**. Therapeutic needs in this area are relatively better met than for autoinflammatory processes and non-histaminergic itch, so we selected a set of inducers that were more relevant to these processes (Table 1).

We generated stock solutions of each inducer we tested by solubilizing them in phosphate-buffered saline (PBS). To treat HMC1.2 cells, we counted them using an automated hemocytometer with a trypan blue dead cell stain and then plated 900,000 cells per well in a 24-well plate in 150 µL media without serum or phenol red before adding 150 µL of serum- and phenol red-free media with inducer at 2× the treatment concentration. We also included a control treatment with PBS. We then incubated cells for 1 h before collecting them for analysis. To recover supernatant, we collected the medium with suspended cells and centrifuged at 300 × g for 5 min.

To treat RBL-2H3 cells, we lifted them with trypsin and counted them, then plated 500,000 cells per well in complete medium in a 24-well plate. We incubated these cells overnight to let them adhere to the plate. Then, we aspirated the complete

medium and washed the cells with PBS before treating them with 300 µL 1× inducer in serum- and phenol-red-free medium. We then incubated these cells for 1 h and collected the supernatant directly (without detaching the cells from the plate) for downstream analysis.

Note: It's important to control for DMSO concentration

Because our tick-derived molecules must be solubilized in DMSO, we also tested the impact of DMSO in our assays. The β-hexosaminidase assay relies on fluorescence excitation at 365 nm. When present, DMSO's absorbance peak at ~380 nm significantly reduces the resulting fluorescent signal. As a result, it's critical to only compare fluorescence readings from wells with equivalent concentrations of DMSO.

Inducer	Target receptor	Treatment concentration	Reference(s)
48/80	MRGPRX2	66.7 µg/mL	**[14][17]**
Substance P	MRGPRX2	10 µM	**[18][19]**
PAMP12 (PAMP9-20)	MRGPRX2	300 µM	**[20]**
5-HT	HTR7 (and other 5HT receptors)	3 µM	**[21][4]**
SLIGRL	PAR2	300 µM	**[22]**
Chloroquine	MRGPRX1	300 µM	**[5][23]**
β-alanine	MRGPRD	100 µM	**[24]**
Histamine	H1R (and other HA receptors)	30 µM	**[25]**

Table 1

List of inducers we tested in an effort to stimulate mast cell degranulation, their respective receptor targets (as reported in the literature), and the treatment concentrations we used.

Because 48/80 is a polymer with non-zero polydispersity, we report its concentration in µg/mL rather than molar units.

β-hexosaminidase degranulation assay

Mast cell granules are filled with effector molecules such as histamine, tryptase, and β-hexosaminidase, which are released into the extracellular space in a process known as degranulation **[2]**. Once degranulation occurs, effector molecules can trigger downstream immune responses to address the stimulus. To detect this process, researchers typically measure the presence of one or more of these mediators in the cell culture supernatant. We chose a β-hexosaminidase (β-hex) activity assay, which is widely used to detect mast cell degranulation. β-hex is an enzyme that cleaves β-glycosidic linkages of N-acetylhexosamine sugars (such as N-acetylglucosamine or N-acetylgalactosamine). This enzymatic activity lets us easily detect degranulation in a cell-based assay. A histamine ELISA is another commonly used option, but we chose

the β-hex assay because enzyme activity assays are faster to implement and easier to perform at high throughput than ELISAs.

To quantify the enzymatic activity of β-hex, we used a β-hexosaminidase activity kit from Cell Biolabs, according to the manufacturer protocol. In brief, we plated 50 µL of sample in triplicate in a 96-well plate. We added 50 µL of a fluorogenic substrate called 4-methyllumbelliferyl-N-acetyl-D-glucosaminide (4-MU-GlcNAc) and incubated the plate for 15 min at 37 °C, protected from light. If β-hex is present, the enzyme will cleave the 4-MU-GlcNAc substrate and release 4-MU, which is fluorescent. Following the incubation period, we added 100 µL of a neutralization buffer to each well and immediately measured fluorescence at 365 nm excitation and 450 nm emission using a plate reader.

Lactate dehydrogenase toxicity assay

When measuring the release of any molecule into the supernatant, it's critical to perform a cell viability assay to ensure that the detected factor's release is the result of a biological process and not unintended cell death, which causes indiscriminate release of many intracellular components. To ensure that our activators induce degranulation and don't cause cell death, we accompanied our β-hex assay with a lactate dehydrogenase (LDH) assay to measure toxicity.

LDH is a constitutively expressed intracellular cytosolic enzyme that's important for anaerobic respiration. Within the cell, it catalyzes the reversible interconversion of pyruvate to lactate using NADH as a cofactor. LDH is not typically secreted, but when cells lyse, LDH is released into the extracellular environment **[26]**. Production of NADH is driven by the addition of excess lactate as a substrate, which in turn drives the reduction of a tetrazolium salt into a colored formazan via diaphorase. We can measure the absorbance of this product over time with a plate reader. An increased rate of NADH production indicates the presence of LDH. By pairing this assay with the β-hex assay, we can draw robust conclusions about whether an activator has induced degranulation.

Using the same supernatant from the β-hex assay, we performed the LDH assay using a kit from Abcam, according to the manufacturer protocol. We used different concentrations of our sample to ensure that the NADH concentrations were in the accurate detection range for an absorbance assay (between 0.1 and 2 absorbance

units). After adding samples to the 96-well plate, we added 50 µL of prepared LDH substrate to each reaction and immediately began measuring absorbance at 450 nm on a plate reader at 37 °C every two minutes for a total of 30 minutes.

Data analysis

We analyzed raw data we collected from a Molecular Devices SpectraMax iD3 plate reader in the β-hex and LDH assays using Jupyter Notebooks.

> These **notebooks** and **associated data** we used to generate the plots in this pub are on GitHub (DOI: 10.5281/zenodo.15041667).

β-hex assay processing and statistical analysis

A notebook for the β-hex assay extracts metadata and well-by-well endpoint data from the raw CSV file and labels them using a sample manifest CSV (produced for each experiment), subtracts background values, generates plots for the standards and samples, and performs a statistical analysis.

LDH assay processing and statistical analysis

A notebook for the LDH assay extracts metadata and well-by-well kinetic data from the raw CSV file, labels them using a sample manifest CSV, and subtracts background values. It then generates a standard curve plot for each time point and an activity curve showing the accumulation of NADH over time for each sample. The user then selects two time points to use for the downstream analysis (default values are 0 and 480 s).

> **Note**
>
> It's best to choose downstream analysis time points in the linear range of the activity curves for all samples.

The downstream analysis first performs a quadratic standard fit at each of the two selected time points and uses these fits to determine the change in NADH concentration in that time period. Next, it parses the dilution factors from the sample names and determines the overall dilution-corrected LDH concentration in each well, and plots these. Finally, the notebook selects the best dilution for each sample to generate a simplified dataset and plot showing only the best dilution and then performs statistical analysis on the processed results.

> **Note**
>
> The calculated NADH concentrations should fall within the linear range of the standard curves and within the range of measured standards (we used a 0–250 µg/mL standard concentration range in 50 µg/mL increments, as recommended by the kit manufacturer). We recommend using the less-diluted sample if multiple dilution readings are within this range.

Inducer panel visualization

To facilitate visualization of the effects of different inducers on degranulation and viability of each cell line, we created an additional notebook that takes the processed CSV files output by β-hex and LDH processing notebooks, standardizes the order of the samples along the x-axis, plots individual replicate data as well as means and error bars, and shows data for each cell line side-by-side.

Dose-response analysis and visualization

We designed a fourth notebook specifically for β-hex and LDH dose-response experiments. This notebook identifies the wells in the dose series, parses their concentrations into floats that all have the same unit (in our case, µg/mL), and generates sigmoid plots [$\log_{10}$(concentration) vs. response]. Then, the notebook performs a four-parameter logistic regression on each dataset to return either the EC_{50} or LC_{50} value for the activator of interest. Finally, the notebook normalizes the response to the range 0–1 and plots both hex and LDH curves on the same plot, enabling us to examine the non-toxic activity window of a given activator.

The results

SHOW ME THE DATA: Access our data on GitHub.

48/80 is toxic in HMC1.2 and RBL-2H3 cells

Mast cells are a major component of immune surveillance and response in the skin, and ticks must contend with this response to feed successfully. We reasoned that mast cells were a likely target of the tick molecules that we predicted were important for long-term blood feeding **[10]**. To screen these molecules for activity *in vitro*, we decided to set up a degranulation assay based on β-hex release in two mast cell model lines, HMC1.2 and RBL-2H3.

To establish the assay, we tested a panel of eight degranulation inducers, including 48/80, for toxicity as well as the ability to induce β-hex release (Figure 1). In our initial tests of this panel of mast cell activators, we observed that only 48/80 induced β-hex release from either HMC1.2 or RBL-2H3 (Figure 1, A). Since 48/80 is reported to be an agonist of MRGPRX2 and an inducer of itch and inflammation in the skin **[14][17]**, we were initially encouraged by this result and planned to test our tick molecules' ability to prevent 48/80-induced degranulation. However, certain unexpected patterns gave us pause. We noticed an atypical dose–response activity relationship. Additionally, when extracting RNA for sequencing from 48/80-treated cells for a parallel project, we observed consistently low yields from these cells, but not from vehicle-treated cells or those treated with alternative inducers. This clued us in that 48/80 might have a toxic effect on our cell lines, even though, to our knowledge, this phenomenon hadn't been described in the literature. We also recognized that the β-hex signal we were observing could be explained equally well by membrane permeabilization caused by cell death as by degranulation.

We decided to test this hypothesis directly with an LDH assay, which measures cell death. LDH is a cytosolic enzyme that is not released during degranulation, or during normal cell activity. Upon testing 48/80 in this assay, we discovered that it causes significant release of LDH (Figure 1, B). Along with our β-hex assay data, these results indicate that 48/80 doesn't selectively induce degranulation in these models but instead causes general membrane permeabilization via an unknown mechanism.

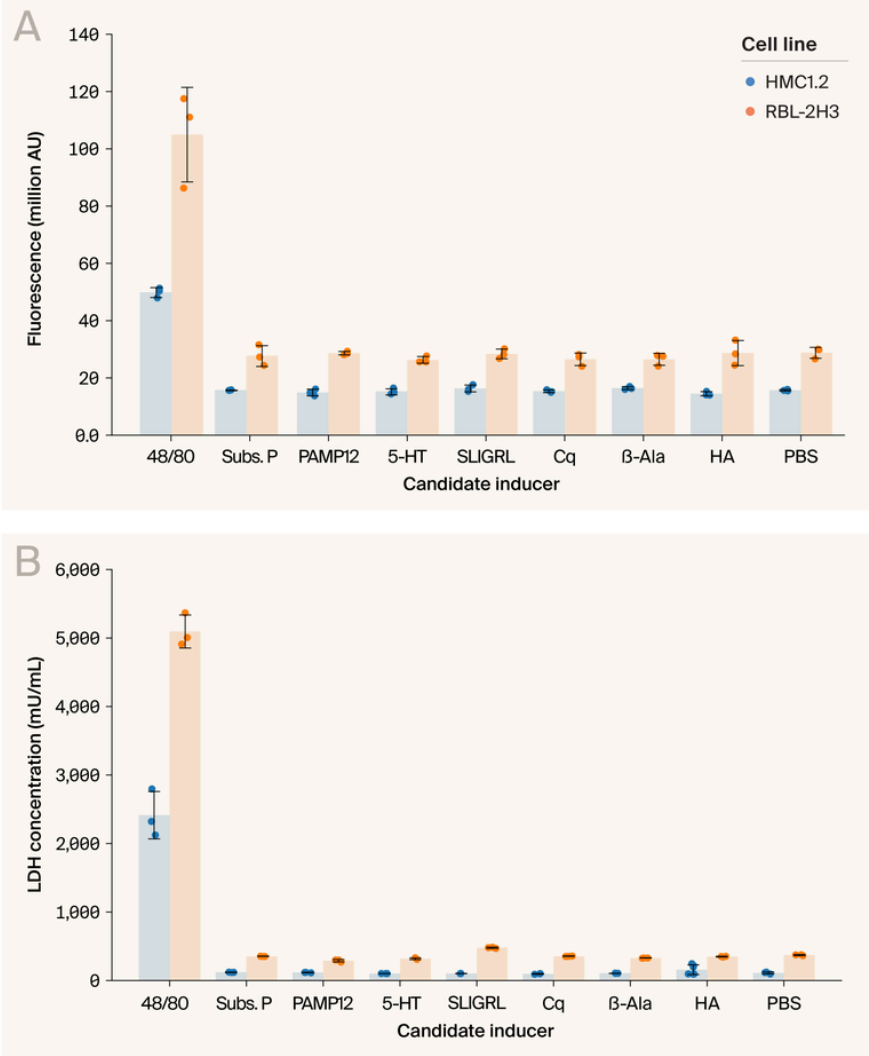

Figure 1

48/80 is the only activator in our panel that can induce β-hex release, but it is also toxic.

We exposed HMC1.2 and RBL-2H3 cells to a panel of inducers for 1 h and analyzed them via (A) β-hex assay to detect degranulation and (B) LDH assay to assess cell viability. We report inducers, their canonical receptor targets, and treatment concentrations in Table 1.

Two of the additional inducers we tested (the peptides Substance P and PAMP12, also known as PAMP9-20) are also reported to function as MRGPRX2 activators like 48/80 **[18][19][20]**. Interestingly, we observed neither β-hex nor LDH release in the presence of high concentrations of these activators. These data suggest that 48/80 toxicity is

not the direct result of MRGPRX2 activation but rather of some other as-yet-undescribed effect.

There is no window to induce degranulation in either cell line with 48/80

We wanted to know whether 48/80 had any useful activity window — concentrations where it could induce degranulation without causing cell lysis. To explore this, we performed dose-response assays with 48/80 in each cell line using the β-hex and LDH assays. We titrated down from our original concentration of 66.7 µg/mL, which we knew induced β-hex and LDH release and which is comparable to concentrations used on these cell lines in the literature (where we've seen groups use up to 1 mg/mL) **[14][17][27][28][29]**.

In RBL-2H3, we observed a small shift in the dose-response curves. Still, it wasn't sufficient to truly separate them such that we could reliably achieve a high β-hex release response without significant toxicity. In HMC1.2, we observed no such separation (Figure 2).

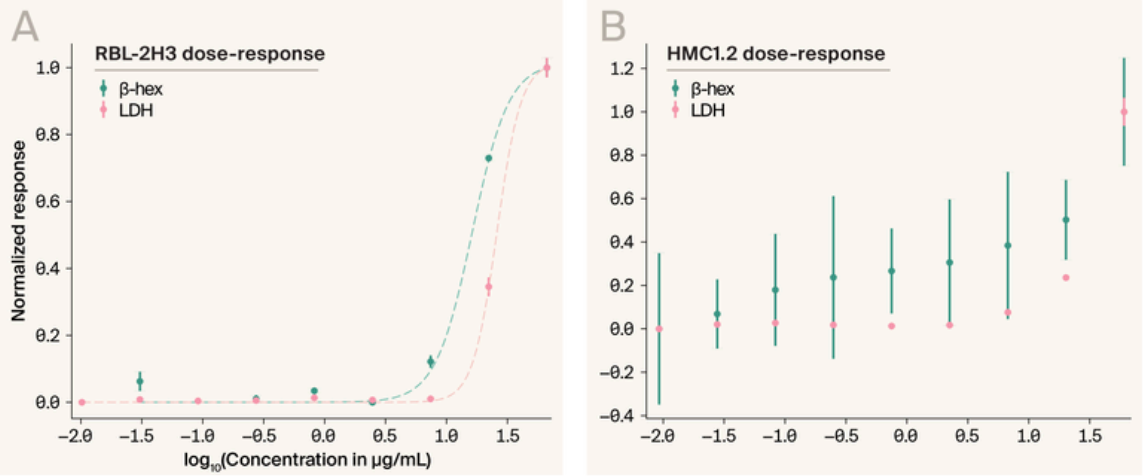

Figure 2

Dose-response analysis of 48/80 β-hex release and cytotoxicity in HMC1.2 and RBL-2H3 cells.

We treated (A) RBL-2H3 and (B) HMC1.2 cells with a concentration series of 48/80 for 1 h and analyzed via hexosaminidase (β-hex) and lactate dehydrogenase (LDH) assays. We calculated and plotted normalized responses. We performed four-parameter logistic regression on data collected from RBL-2H3 and LC_{50} (25.9 µg/mL) and calculated degranulation EC_{50} (16.3 µg/mL) values. Logistic regression failed for data we collected from HMC1.2.

We also observed that HMC1.2 cells released significantly less β-hex than RBL-2H3 (Figure 1 and Figure 2). This is consistent with reports that HMC1.2 cells are poorly granulated **[14]** and resulted in greater relative variability in our measurement of β-hex release from HMC1.2 compared to RBL-2H3.

From these experiments, we concluded that β-hex released by 48/80 is an artifact of membrane permeabilization and not the result of a biological process induced by this compound.

Key takeaways

Using β-hexosaminidase and LDH release assays, we demonstrated that 48/80, a commonly used mast cell activator, is toxic in HMC1.2 and RBL-2H3 cell lines, two

widely used mast cell models. We found no concentration of 48/80 that we could use to induce degranulation without causing toxicity. Researchers studying mast cell biology should carefully consider whether 48/80 is an appropriate mast cell activator, and experiments that use 48/80 to activate mast cells should be interpreted with caution.

Caveats

We only tested two cell lines

While we reproducibly observed toxicity in cells we treated with 48/80, these observations occurred in two specific cell lines, HMC1.2 and RBL-2H3. We don't definitively know whether the same effect occurs in other *in vitro* mast cell models, such as LAD2 cells, or *in vivo*. If others have tested this, especially if someone has identified 48/80 treatment conditions that can induce non-toxic degranulation of mast cells either *in vitro* or *in vivo*, we'd be excited to learn about it.

There may be non-toxic ways to induce degranulation

Many compounds activate signaling pathways that lead to degranulation. We selected a set that is biologically relevant to the molecules we're testing and the biology we're studying, but we're not claiming that there are no non-toxic inducers of degranulation in these cell lines. In fact, we'd love to know if other groups are aware of any.

Mechanism unclear

We also don't know the mechanism of 48/80's toxicity, although our data indicate that it isn't directly related to 48/80's canonical activity as an MRGPRX2 agonist. In further support of this hypothesis, there are also reports of 48/80 being used to cause outer membrane permeabilization in *Pseudomonas aeruginosa* **[30]** and K^+ efflux in mammalian mast cells **[31]**, suggesting that it may non-specifically permeabilize the

membrane. Such an effect would be consistent with 48/80's amphipathic chemical structure (Figure 3).

Next steps

Because none of the other inducers we're interested in could induce degranulation, we decided to put the mast cell degranulation assay on ice for now. As an alternative strategy to measure activation of mast cells and other skin-resident immune cells, we're turning to a qPCR-based approach to measure the expression of inflammatory cytokines. This will help us understand whether our activators of interest are producing a different measurable response in these cells at non-lethal concentrations. It will also allow us to study the impact of compounds we're testing against a broader range of inflammatory mechanisms, rather than just mast cell degranulation.

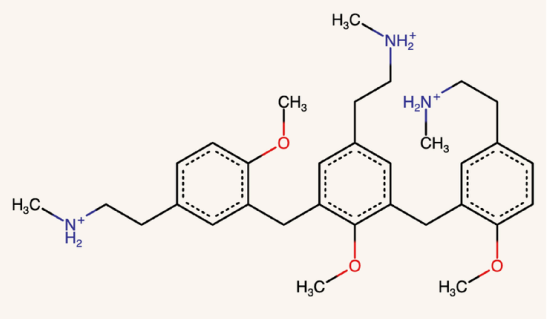

Figure 3

Chemical structure of a 48/80 tripolymer at neutral pH.

Each monomer unit of 48/80 contains a hydrophobic substituted phenyl ring and a positively charged secondary amine. Other amphipathic chemicals, including detergents, cell-penetrating peptides, and antimicrobial peptides, are known to compromise the integrity of cell membranes.

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