Measurement of Accumulation of Small Molecules into Gram-negative Bacteria

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Abstract

Some of the most dangerous bacterial pathogens (Gram-negative and mycobacteria) deploy a formidable secondary membrane barrier to reduce the influx of exogenous molecules. For Gram-negative bacteria, this second exterior membrane is known as the Outer Membrane (OM) and its unique composition is generally what restricts the passive permeation of small molecules into bacterial cells. While it is well appreciated that the OM is a principal determinant of small molecule permeation, it has proven to be challenging to assess this feature in a robust and quantitative way. Herein, we describe the development of the Bacterial Chloro-Alkane Penetration Assay (BaCAPA), which employs the use of a genetically encoded protein called HaloTag, to measure the uptake and accumulation of molecules into Gram-negative bacteria. Directing the localization of the HaloTag protein to either the cytoplasm or periplasm of *Escherichia coli* (*E. coli*) enabled a compartmental analysis of permeation across individual cell membranes. Significantly, we also showed that BaCAPA can be used to analyze the permeation of molecules within the phagocytes of macrophages, which may prove critical in the analysis of intracellular pathogens. Together, our results show that BaCAPA can effectively report on the accumulation of molecules into bacterial cells and we anticipate that this will widen the set of tools available to generally measure permeability into bacterial cells.

Introduction

The rise in incidence of drug-resistant bacterial infections poses a tremendous challenge for healthcare systems throughout the world.[1-3] While recent efforts have led to the discovery of new therapeutic leads with promising clinical potential,[4-10] there is a continued need to strengthen the antibiotic pipeline and to find antibiotics with narrow spectrum activities to reduce off target impact on gut commensal bacteria. Most antibiotics must enter the bacterial cell to impart their biological effects, which includes permeating through a lipid bilayer. For Gram-negative bacteria, there is an additional challenge due to the presence of an asymmetrical bilayer known as the outer membrane (OM).[11-12] For some agents (e.g., β-lactam antibiotics), permeation through OM-anchored porins can potentially provide an access pathway to the periplasm.[13] However, many of the most potent antibiotics have cytosolic targets, and, therefore to be effective they must also permeate through the inner plasma membrane. Additionally, a reduction in the amount of antibiotics accumulating in Gram-negative bacteria can be further modulated by the active removal of drugs that reach the periplasm by efflux pumps that recognize broad structural motifs.[14-15]

Despite the fact that drug permeation is well recognized as a major bottleneck for antibiotic efficacy and directly tied to drug discovery efforts[16-17], there are few methods developed to measure the accumulation of small molecules in Gram-negative bacteria.[18] As such, gaps remain in our fundamental understanding of the molecular determinants of permeability. Direct methods of measuring drug uptake and retention

can be extremely valuable in providing insight into drug accumulation profiles. The Hergenrother group[19] adopted a protocol[20] that uses liquid chromatography with tandem mass spectrometry (LC-MS/MS) to expand the analysis of drug permeation across 180 diverse molecules in *Escherichia coli* (*E. coli*). A principal finding from these investigations was that primary amines as a functional group are privileged moieties in promoting accumulation in Gram-negative bacteria. More recent studies leveraged biorthogonal reactions to probe the accumulation to distinct compartments within *E. coli*.[21-22] We sought to complement existing strategies by retaining the targeted compartmentalization of detection while simplifying the mode of accumulation readout.

We, herein, disclose an assay that is adapted from the chloroalkane penetration assay (CAPA) developed by the Kritzer lab[23], which is now widely used to measure the accumulation of molecules into mammalian cells. This method relies on the genetically encoded HaloTag[24], a mutant form of a bacterial haloalkane dehalogenase that facilitates covalent bond formation with chloroalkane-linked ligands (**Figure 1**). Previous efforts utilizing the HaloTag protein within bacteria focused on the visualization of protein fusions[25-26] and the role of a cationic peptide on membrane permeabilization.[27] In building on these precedents, we sought to develop a Bacterial Chloroalkane Penetration Assay (BaCAPA) to measure the apparent accumulation of molecules into bacterial cells. The assay is based on the expression of the HaloTag protein inside bacterial cells, which are exposed to molecules of interest linked to a chloroalkane tag. If the molecules reach the HaloTag proteins inside the bacteria, they will be covalently bound to the protein. This pulse step is followed by a chase step with a fluorophore-

linked chloroalkane. In the absence of drug accumulation, HaloTag active sites remain available to react with the fluorophore. Conversely, when a large fraction of molecules of interest permeate the cell membranes and reach the HaloTag proteins, there will be reduced sites available to react with the fluorophore. Therefore, a decrease in cellular fluorescence signal should be reflective of the apparent drug accumulation into bacterial cells. We sought to develop BaCAPA to readily and robustly measure accumulation in *E. coli*. The ultimate goal is to establish structural determinants that favor permeation of small molecules across the cellular envelope of Gram-negative bacteria to discover privileged antibiotic scaffolds.

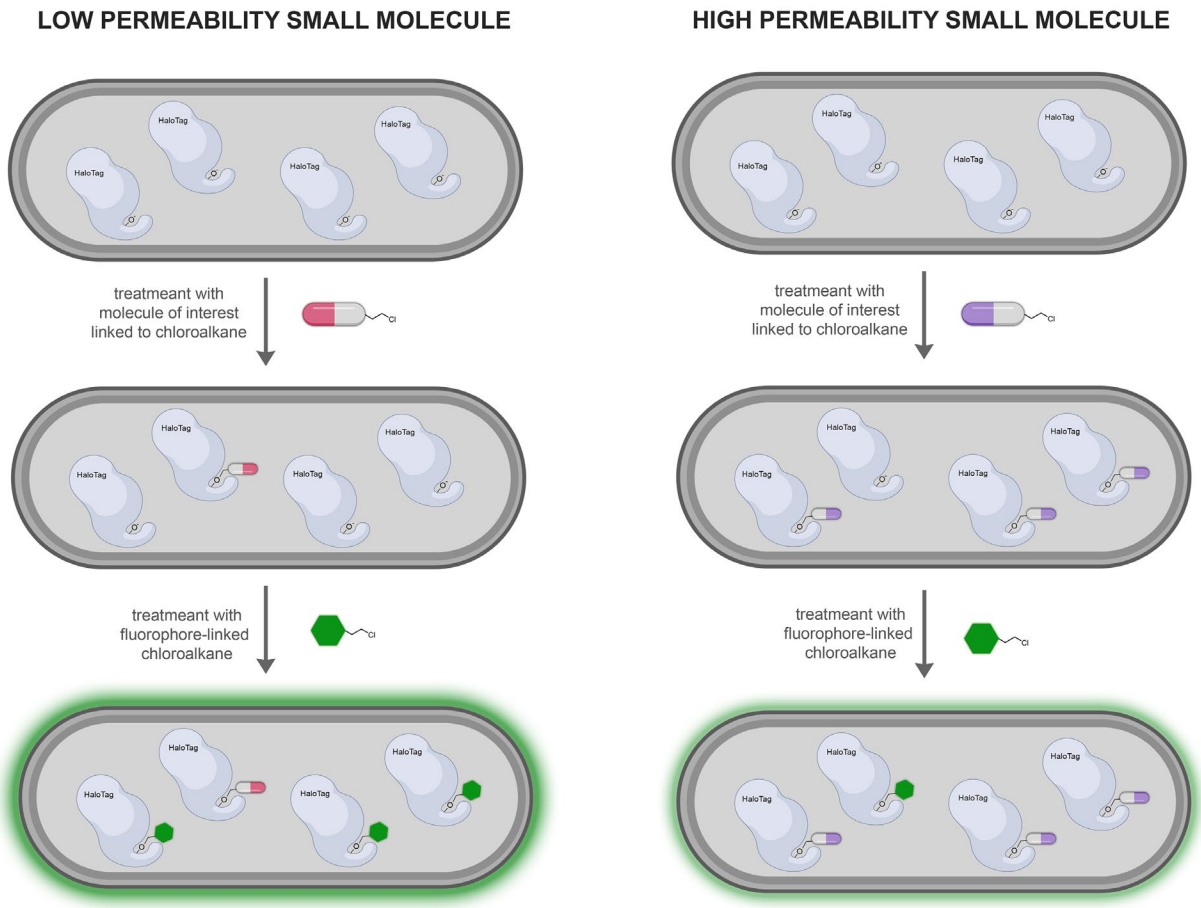

Figure 1. Schematic representation of BaCAPA. Bacterial cells expressing HaloTag will covalently react with molecules of interest, thus reducing the number of sites available to react with the fluorophore.

At first, we aimed to evaluate the fluorescence levels of bacterial cells that express HaloTag proteins in the cytosol by encoding the protein on an inducible plasmid. Bacterial cells carrying the HaloTag-expressing plasmid were grown to mid-log, induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG), and incubated with a fluorophore modified chloroalkane. Cytosolic HaloTag should promote the formation of a covalent bond with the fluorophore-linked chloroalkane tail (**Figure 2A**). Total labeling levels of bacterial cells can then be quantified using flow cytometry. To establish the first step in the BaCAPA, bacterial cells were treated with Rhodamine 110 modified with chloroalkane (**R110cl**). As expected, a large increase in cellular fluorescence was observed in a dose dependent manner with increasing levels of IPTG (**Figure 2B**). These results are suggestive of a HaloTag-mediated reporting on the accumulation of **R110cl** into bacterial cells. Appreciating that the fluorophore itself could be subject to the same barrier elements that are inherent to *E. coli*, we also evaluated the labeling of bacterial cells with a smaller fluorophore, namely coumarin (**COMcl**). We projected that the physiochemical properties of these dyes could alter permeation into bacterial cells due to differences in size and charge between the two dyes. While it was clear that coumarin could also covalently modify HaloTag-expressing bacterial cells, the relative

fluorescence increase was much more modest in cells treated with **COMcl** (**Figure 2C**). Therefore, **R110cl** was chosen as the reporter dye for all subsequent studies.

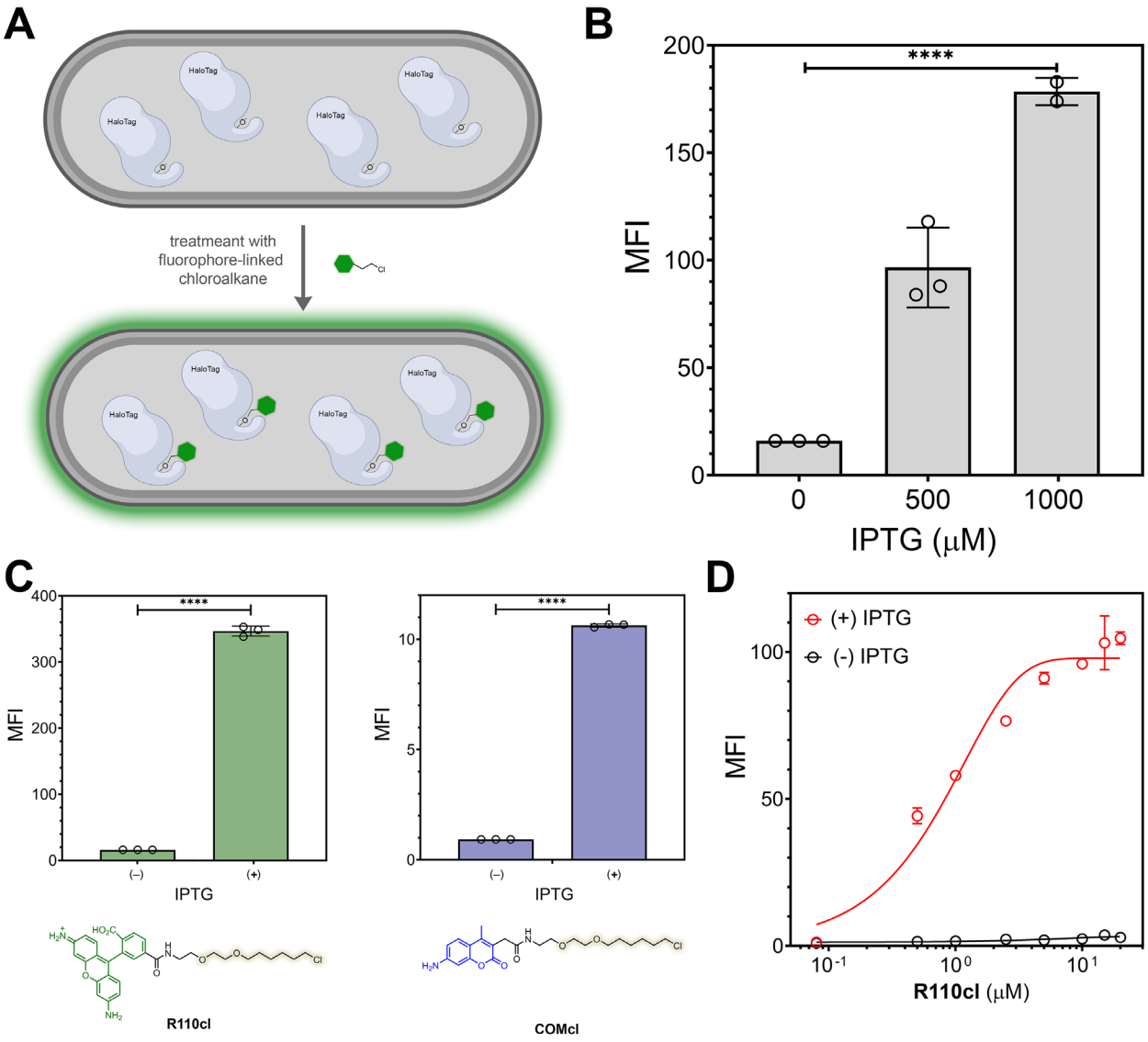

Figure 2. (A) Schematic representation of reaction of a fluorophore-linked to chloroalkane with HaloTag inside a bacterial cell. (B) Flow cytometry analysis of *E. coli* treated with various concentration of IPTG, followed by an incubation period with 5 μM

of **R110cl**. (C) Flow cytometry analysis of *E. coli* treated with either 5 μM of **R110cl** or **COMcl**. (D) Flow cytometry analysis of *E. coli* treated with various concentrations of **R110cl** in the presence and absence of IPTG induction. Data are represented as mean +/- SD (n = 3). *P*-values were determined by a two-tailed *t*-test (* denotes a *p*-value < 0.05, ** < 0.01, ***<0.001, ns = not significant).

To empirically establish the dye concentration that optimizes the signal output for the assay, bacterial cells were treated with a range of **R110cl** concentrations (**Figure 2D**). The results showed that 0.5 μM is sufficient to yield fluorescence levels that were nearly 50% of the maximum and by 5 μM the fluorescence signals were reaching saturation levels. For these reasons, all subsequent assays were carried out using 5 μM of **R110cl**. We posed that it should be possible to analyze HaloTag labeling with **R110cl** from the whole cellular proteome because of the selectivity of this enzymatic reaction. To this end, bacterial cells were treated similar to the previous experiments, subjected to separation on an SDS-PAGE, and the gel was imaged using a fluorescent filter. As expected, there is a clear band that corresponds to the molecular weight of HaloTag (**Figure S1**), which strongly indicate that the signals observed from the flow cytometry analysis are representative of HaloTag covalently bound with **R110cl** inside bacterial cells. Cells were also imaged using confocal microscopy and the labelling pattern was consistent with the projected labeling of HaloTag (**Figure S2**). Together, these results serve to establish the working parameters for BaCAPA.

It is well appreciated that the OM is the major barrier to the permeation of small molecules.[28] Considerable efforts have been devoted to the discovery of molecules that disrupt the OM barrier as antibiotic adjuvants with the goal of potentiating them and/or circumventing resistance.[29-31] Therefore, these agents hold the promise of synergistically enhancing the permeation of potential antibiotics in a broad and, potentially, impactful way. This class of molecules is best represented by the Gram-negative specific antibiotic polymyxin B. More specifically, a fragment called polymyxin B nonapeptide (PMBN), which lacks the fatty acid tail, has permeabilizing properties at concentrations that are non-toxic to bacterial cells.[32] For example, PMBN demonstrated the ability to protect mice against Gram-negative bacteria when dosed with erythromycin.[33] We reasoned that co-treatment of cells with PMBN and **R110cl** should introduce a greater level of **R110cl** to bacterial cells, thus resulting in higher fluorescence levels (**Figure 3A**). Indeed, a concentration-dependent increase in cellular fluorescence was observed and the fluorescence levels of cells treated with 10 μM of PMBN was double than that of cells not treated with PMBN. Moreover, no loss of bacterial viability was observed at the concentrations of PMBN tested (**Figure S3**).

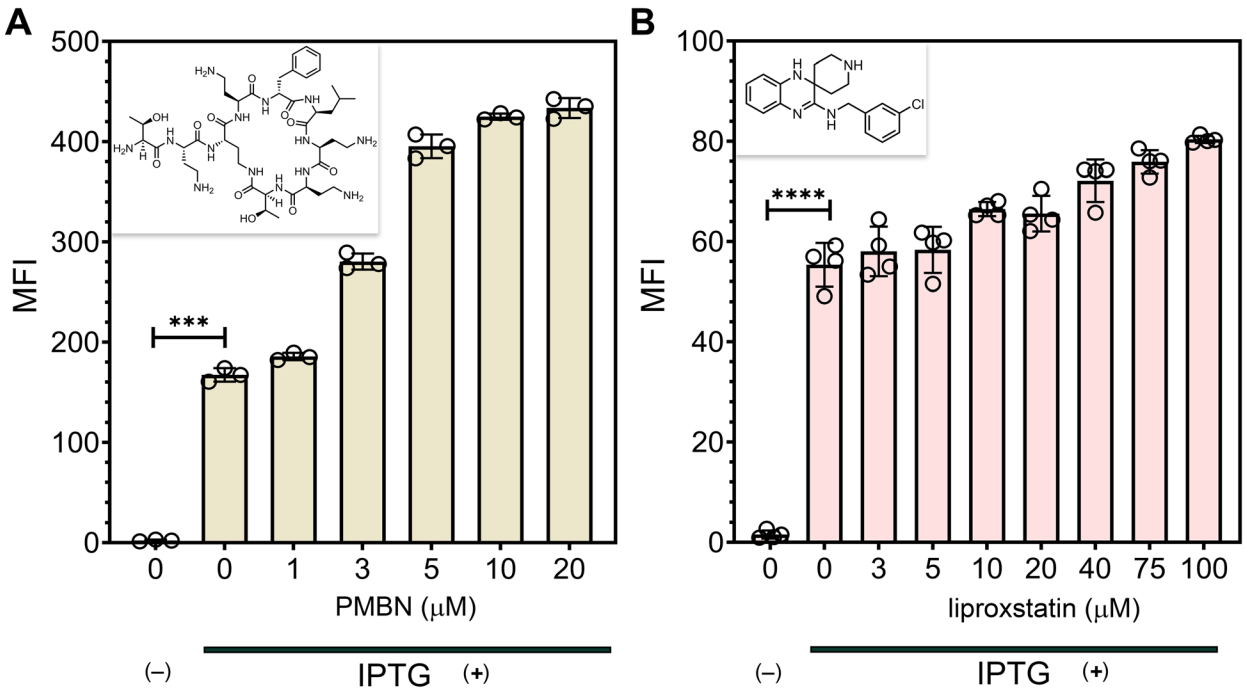

Figure 3. Flow cytometry analysis of *E. coli* treated with various concentrations of PMBN (A) or liproxstatin (B), followed by a treatment with 5 μM of **R110cl** in the presence and absence of IPTG induction. Data are represented as mean +/- SD (n = 3). *P*-values were determined by a two-tailed *t*-test (* denotes a *p*-value < 0.05, ** < 0.01, ***<0.001, ns = not significant).

A recent screening campaign by the Brown laboratory against a library of 140,000 diverse compounds revealed liproxstatin as a promising new disruptor of *E. coli* OM.[30] Similar to PMBN, an increase in cellular fluorescence was observed when liproxstatin was co-incubated with **R110cl** (**Figure 3B**). These results demonstrate the potential of BaCAPA to be leveraged, more generally, to report on molecules that disrupt the outer membrane of Gram-negative bacteria. In an effort to test the assay compatibility with high-throughput screening platforms, we questioned if the cellular fluorescence levels

could be measured *via* a plate reader rather than flow cytometer. Our results showed that the assay could readily report on HaloTag-mediated fluorescence (**Figure S4**). While there was a decrease in signal-to-noise, the signal strength in the presence of IPTG was well above that in the absence of IPTG. We are currently exploring the full compatibility of the assay to be used to screen for molecules that increase cellular permeability in Gram-negative bacteria.

Next, we set out to show that BaCAPA could be implemented in a smooth *E. coli* strain (ATCC 25922) that contains the complete set of lipopolysaccharides on the surface. In contrast to K-12 rough bacteria, smooth *E. coli* strains can potentially have altered permeability. We recently demonstrated that the surface of rough *E. coli* had higher accessibility to molecules than smooth *E. coli*, presumably due to the steric blockade provided by *O*-antigens.[34] Similarly, it is possible that the *O*-antigens may contribute to the overall permeation profile of small molecules considering that LPS chains have been proposed to occlude porin channels via/by steric shielding.[35] Therefore, we set out to establish the feasibility of analyzing BaCAPA in smooth WT *E. coli* in a similar manner to that of the K-12 *E.* coli. When induced and non-induced WT *E.coli* cells were treated with **R110cl**, we observed a HaloTag dependent increase in fluorescence; therefore, these results confirmed that the permeability assay can be readily implemented in a WT strain (**Figure S5**). Additionally, we posed that it may be possible to take advantage of directed localization tags to specifically probe the accumulation of molecules within individual compartments in *E. coli*. To test this, an *N*-terminus fusion of a DsbA signal peptide was added to HaloTag to transport it to the periplasmic space.[36]

While we observed minimal fluorescence signals in *E. coli* BL21(DE3), high fluorescence levels were observed in Lemo21 (DE3) (**Figure S6**). Expression in Lemo21 cells likely afforded a more controlled expression level that may have favorably modulated the folding of HaloTag. Additionally, confocal microscopy established that the DsbA-HaloTag labeling pattern is more consistent with the expected localization within the periplasmic space compared to that of the cytoplasmic HaloTag (**Figure S7**).

We then set out to test the ability of BaCAPA to report on apparent accumulation of small molecules past the cellular envelope. In this format, (non-fluorescent) small molecules modified with the chloroalkane are incubated with the bacterial cells expressing HaloTag first (pulse step), which is then followed by a treatment step with the dye **R110cl** (chase step). The initial test was performed with a simple amino-ligand (**1**). A concentration scan of the small molecule revealed that nearly background fluorescence levels were observed at 12 μM with an EC_{50} ~ 5 μM (**Figure S8**). These results suggest that BaCAPA can robustly and efficiently report on the apparent accumulation of small molecules into bacterial cells. We next wanted to evaluate how subtle changes to the chemical structure of the chase molecule could potentially impact its permeability into the bacterial cells. We decided to test how BaCAPA can be leveraged to evaluate the impact of methylation of a primary amine on relative accumulation in *E. coli* (**Figure 4**). The Hergenrother lab previously demonstrated that the inclusion of a primary amine resulted in considerably high levels of accumulation in *E. coli* relative to any other methylation state across a series of molecules (although there were some deviations observed).[19] Interestingly, our data revealed that within our

series of molecules, there was minimal difference in relative accumulation across the primary, secondary, or tertiary amino group (compounds **2**-**4**) (**Figure 4**). There was, however, an observed and statistically significant lowered accumulation level with the quaternary ammonium derivative (**5**). We pose that several factors could potentially explain our observations. First, the mode of permeation is likely to be an important molecular determinant towards the accumulation into the bacterial cells and its subsequent potential efflux. Therefore, the **2**-**4** series could be entering through a different route than prior molecules investigated by the Hergenrother group. Alternatively, we reason that the series chosen here has the potential for an internal hydrogen bond,[37] of which may alter the desolvation energetics, ultimately impacting permeation profiles across low dielectric environments.

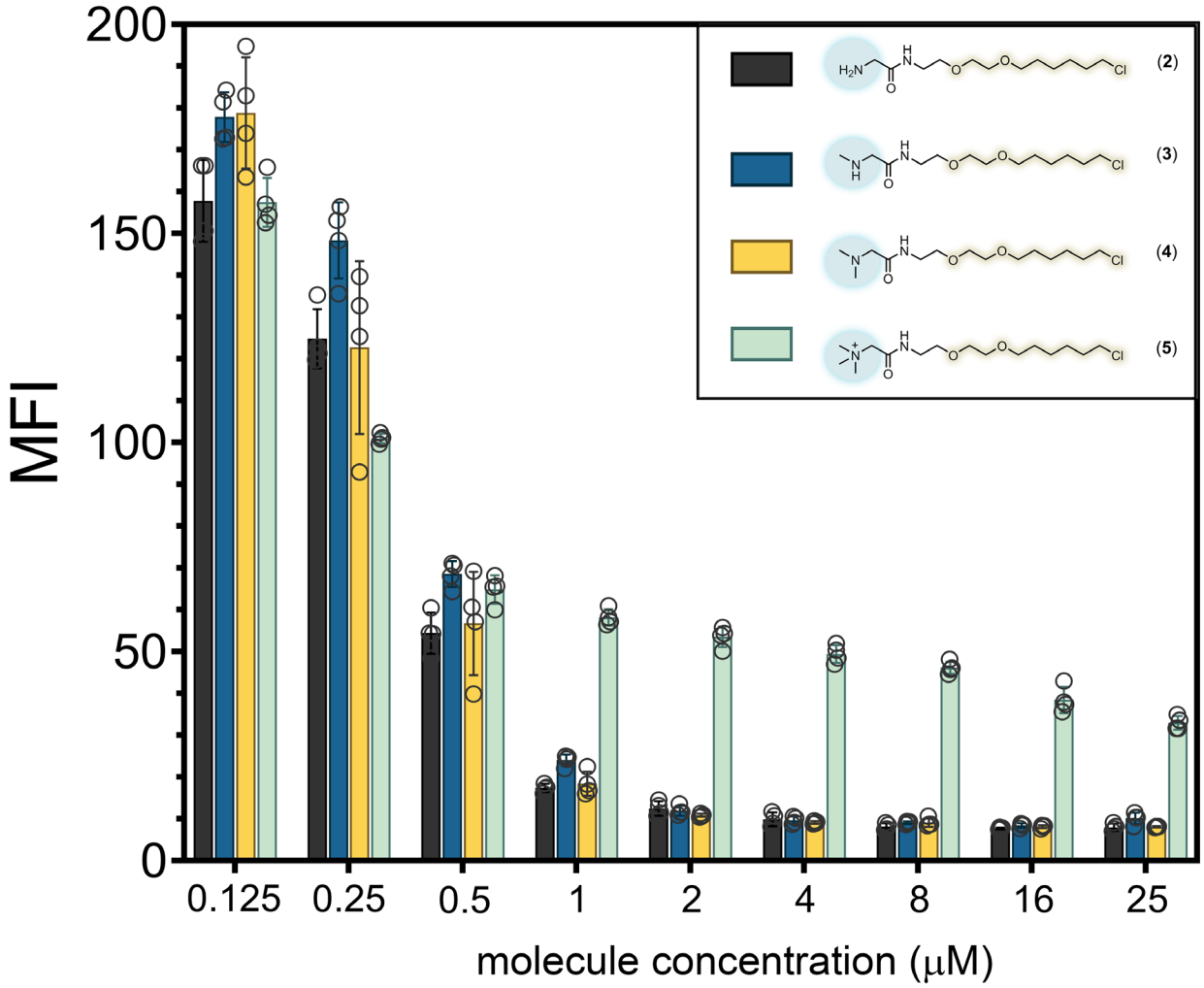

Figure 4. Flow cytometry analysis of *E. coli* treated with various concentrations of molecules **2**-**5**, followed by a treatment with 5 μM of **R110cl** in the presence and absence of IPTG induction. Data are represented as mean +/- SD (n = 3).

To expand the application of BaCAPA for studying the permeability in *E. coli*, a group of molecules were synthesized based on the antibiotic ciprofloxacin decorated with that chloroalkane tag. After the discovery of quinolones as potent inhibitors of DNA replication in bacteria, this molecular scaffold was vastly explored in search of more potent analogs, which yielded clinically important agents like norfloxacin, sparfloxacin,

levofloxacin, moxifloxacin, gatifloxacin.[38] A large fraction of ciprofloxacin analogs involve derivatives to the piperazine moiety, which has shown to be tolerant of functional group manipulations. Therefore, we made two analogs that were built from modifications of the secondary amine for installation of the chloroalkane linker. In one analog, the secondary amine is converted to an amide (**6**) and another where the modification was made with the goal of retaining an ionizable amino group (**7**). A third molecule was synthesized in which the chloroalkane was, instead, coupled onto the carboxylic acid on the parent molecule (**8**). The free carboxylic acid is known to be crucial for the antimicrobial activity, and, therefore, we did not expect that this molecule would have biological activity.[39] Nonetheless, it provided an opportunity to assess how the permeation could be modulated by making a derivative that should have a net positive charge. Our results showed that retention of an ionizable amine group in (**7**) resulted in improved apparent permeation (EC_{50} = 6.5 μM) relative to the similarly structured analog with an amide (**6**), which had an EC_{50} = 42.2 μM (**Figure 5**). Interestingly, we found that these two derivatives had the same MIC value of 0.5 μg/mL. These results highlight the deviation that can arise from using MIC values as a proxy for relative permeation of molecules into Gram-negative bacteria. Installment of the chloroalkane ligand in (**8**) led a significant reduction in EC_{50} (0.5 μM); nonetheless, the MIC value increased 8-fold to 4 μg/mL.

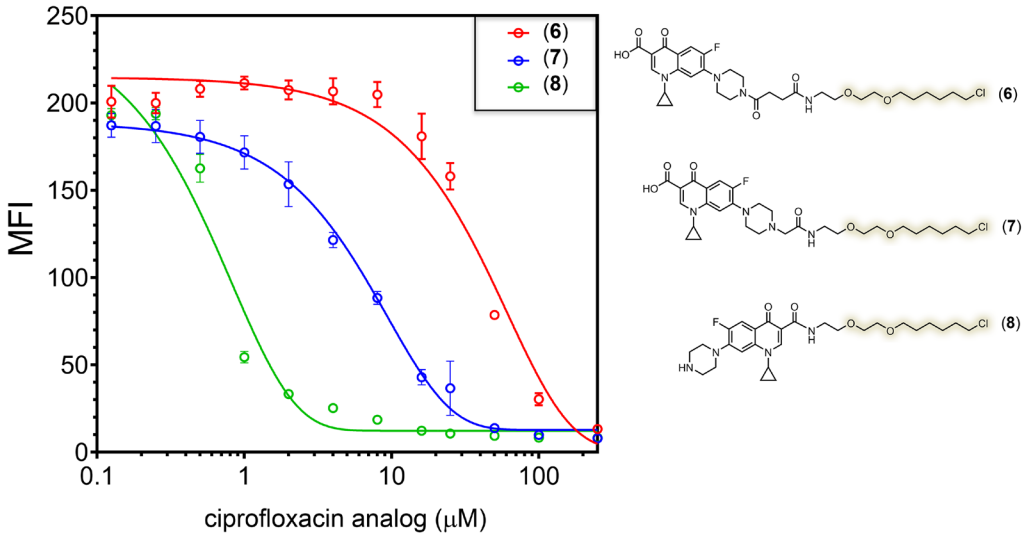

Figure 5. Flow cytometry analysis of *E. coli* treated with various concentrations of molecules **6-8**, followed by a treatment with 5 μM of **R110cl** in the presence and absence of IPTG induction. Data are represented as mean +/- SD (n = 3).

Finally, we hypothesized that BaCAPA should also be operational in probing accumulation of small molecules into bacteria residing inside host mammalian cells (**Figure 6A**). Given the CAPA previously showed low background labeling of mammalian cells in the absence of HaloTag expression, the same should be expected when those cells are infected with bacteria.[23, 40-42] The principal reason to establish the feasibility of BaCAPA inside mammalian cells is that a large fraction of serious human pathogens (e.g., *Mycobacterium tuberculosis, Salmonella enterica*, *Chlamydia trachomatis*, and *Neisseria gonorrhea*) can survive (sometimes exclusively) inside host cells.[43] Moreover, extracellular bacteria can also temporarily reside inside host cells to avoid the action of antibiotics and to promote tissue dissemination.[44-45] The mammalian

plasma membrane can compound the challenges in drug accumulation by introducing an additional barrier. Recent advances in the measurement of accumulation of drugs into bacterial cells have not been extended to intracellular bacteria in mammalian cells. To demonstrate a possible iteration of this type of analysis, we set out to show that there was specific labeling of bacterial cells inside macrophages. Prior to exposure to macrophages, HaloTag protein expression was induced with IPTG and after phagocytosis of bacterial cells mammalian cells were treated with **R110cl** (**Figure 6A**). Our results revealed that there was a large increase in fluorescence levels when macrophages were incubated with HaloTag expressing bacterial cells (**Figure 6B**). The absence of HaloTag induction and macrophages alone showed background levels of fluorescence. The same bacterial cells were also subjected to treatment with **R110cl** (with no macrophage incubation) and were found to have, as expected, high levels of fluorescence when induced with IPTG (**Figure S9**). Confocal microscopy confirmed that cellular fluorescence was exclusively localized to bacteria cells (**Figure 6C**). These results establish that BaCAPA has the potential to achieve an unprecedented level of analysis in drug accumulation into bacteria residing inside mammalian cells. In the future, we will further explore accumulation profiles in a wide range of intracellular bacterial pathogens and define how the permeation profile compares with extracellular conditions.

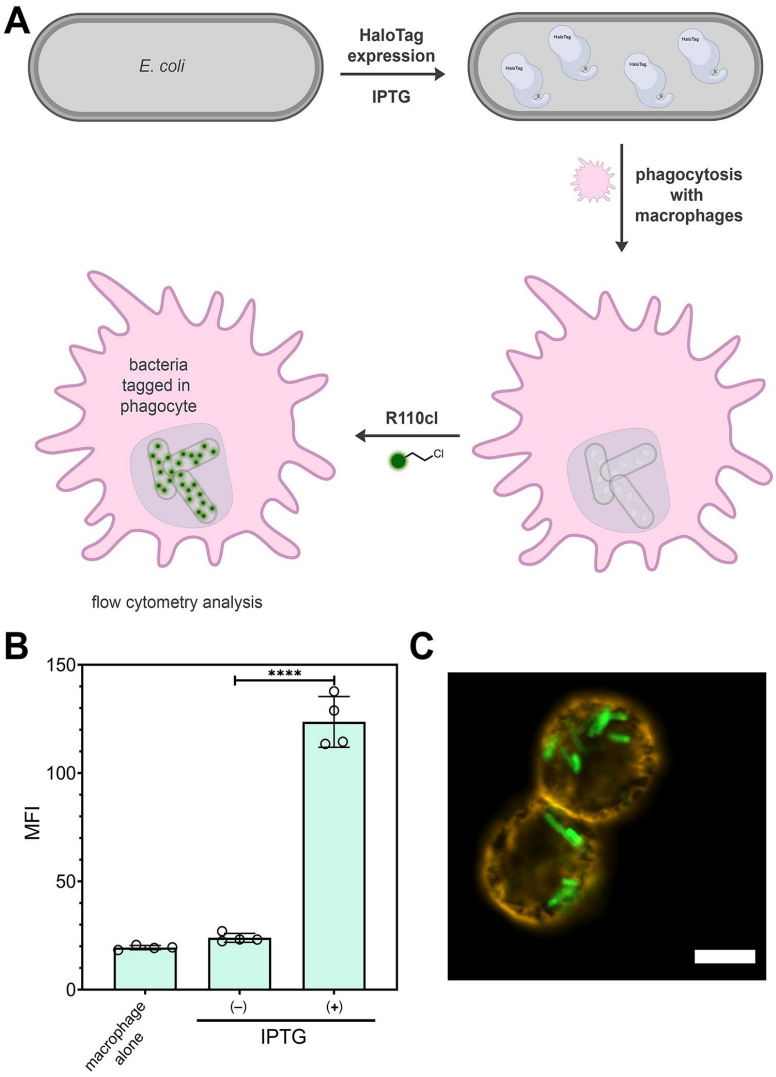

Figure 6. (A) Schematic representation of BaCAPA of bacteria inside mammalian cells. (B) Flow cytometry analysis of J774 cells alone, or J774 cells phagocytosed *E. coli* in the presence and absence of IPTG induction treated with 5 μM of **R110cl**. Data are represented as mean +/- SD (n = 3). *P*-values were determined by a two-tailed *t*-test (* denotes a *p*-value < 0.05, ** < 0.01, ***<0.001, ns = not significant). (C) Confocal microscopy images of J774 macrophages with phagocytosed *E. coli* that were treated with 5 μM of **R110cl**. Cells were fixed with formaldehyde and treated with tetramethyl-

rhodamine-tagged WTA (5 μg/mL) for 30 min. Shown is the overlay of the channels corresponding to rhodamine 110 and tetramethyl-rhodamine. Scale bar = 10 μm.

In conclusion, we have shown that BaCAPA is able to readily report on the accumulation of small molecules in *E. coli via* irreversible covalent bond formation with HaloTag. The expression of HaloTag in the cytoplasm or periplasm of *E. coli* resulted in labeling that was specific to localization of the protein. Fluorescence signals with a rhodamine-based dye proved to be superior to coumarin, thus resulting in sensitive measurements. We first demonstrated that BaCAPA can also be adopted to establish the disruption of the outer membrane of E. coli as demonstrated by PMBN and liproxstatin. Next, we assembled a library of small molecules containing an amino group with varying levels of methyl modifications and found that for this group of molecules, the methylation level was mostly independent of the apparent accumulation levels. We also synthesized a group of ciprofloxacin analogs and observed that there was a wide range of apparent accumulation based on the structural modifications. Most significantly, there was a prominent discord between apparent accumulation levels and the MIC values of these modified ciprofloxacin. These results highlight the potential challenges in using MIC values as a proxy for drug accumulation, which can confound assignments for structure-activity relationships. Finally, we showed that bacteria-expressing HaloTag could be specifically labeled inside macrophages in a manner that is compatible with BaCAPA. Together, our results establish BaCAPA as a robust and, potentially, readily adoptable fluorescence-based reporter for the accumulation of molecules into bacterial cells.

Supporting Information

Additional figures, tables, and materials/methods are included in the supporting information file.

Acknowledgement

This study was supported by the NIH grant GM124893-01 (M.M.P.). We acknowledge the Keck Center for Cellular Imaging for the usage of the Zeiss 980 microscopy system (PI:AP; NIH-OD025156).

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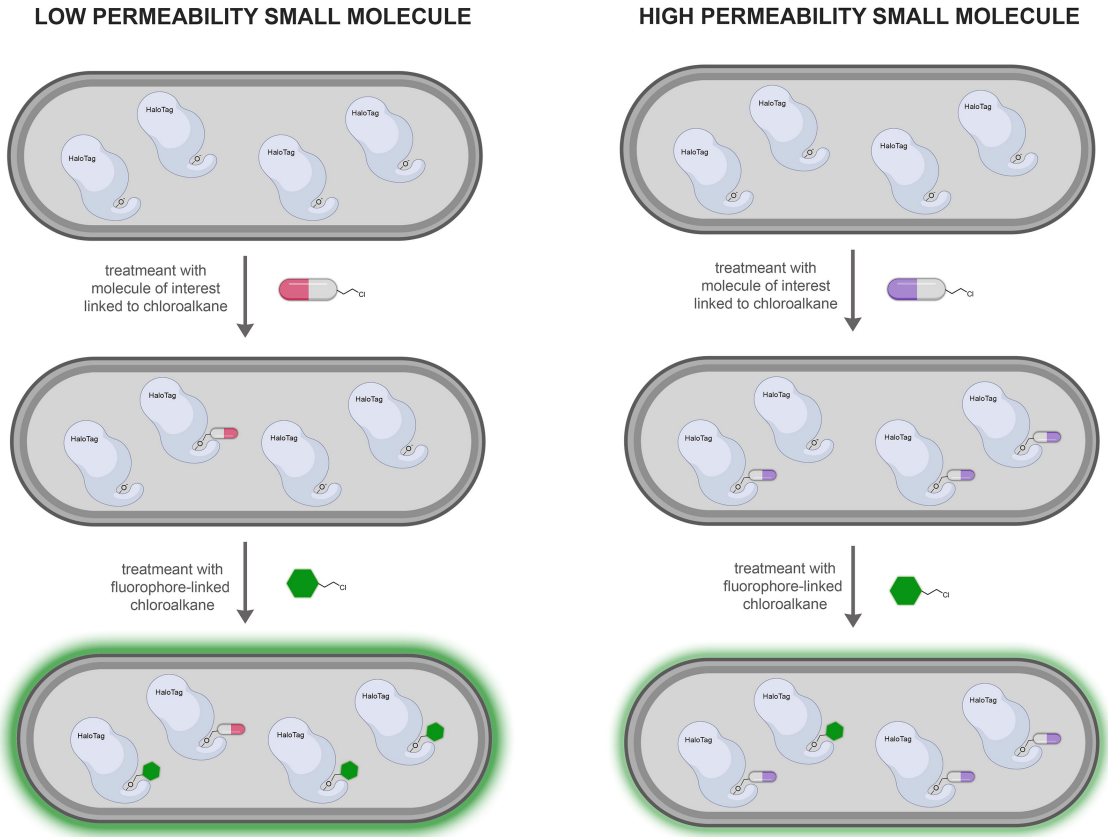
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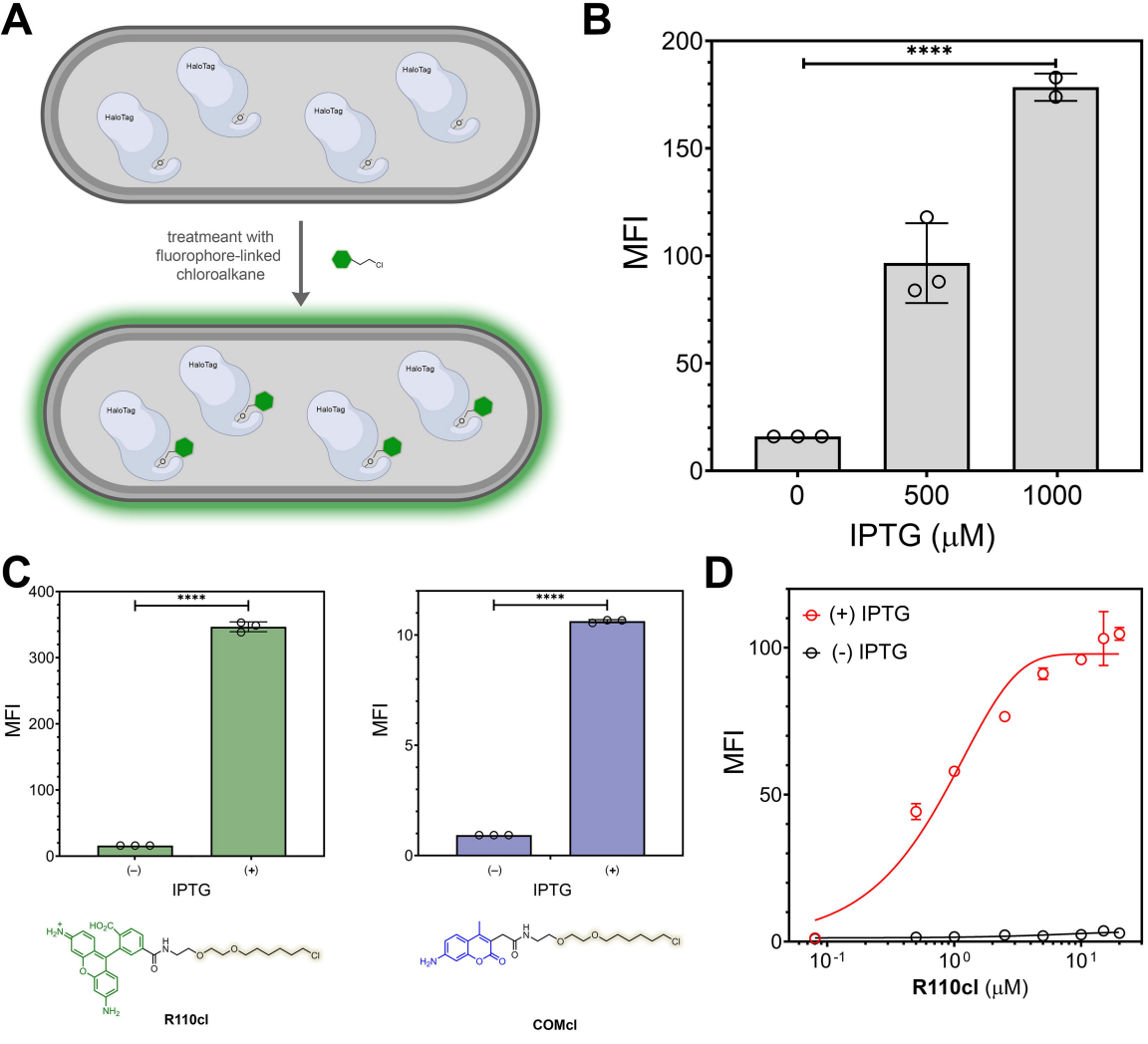
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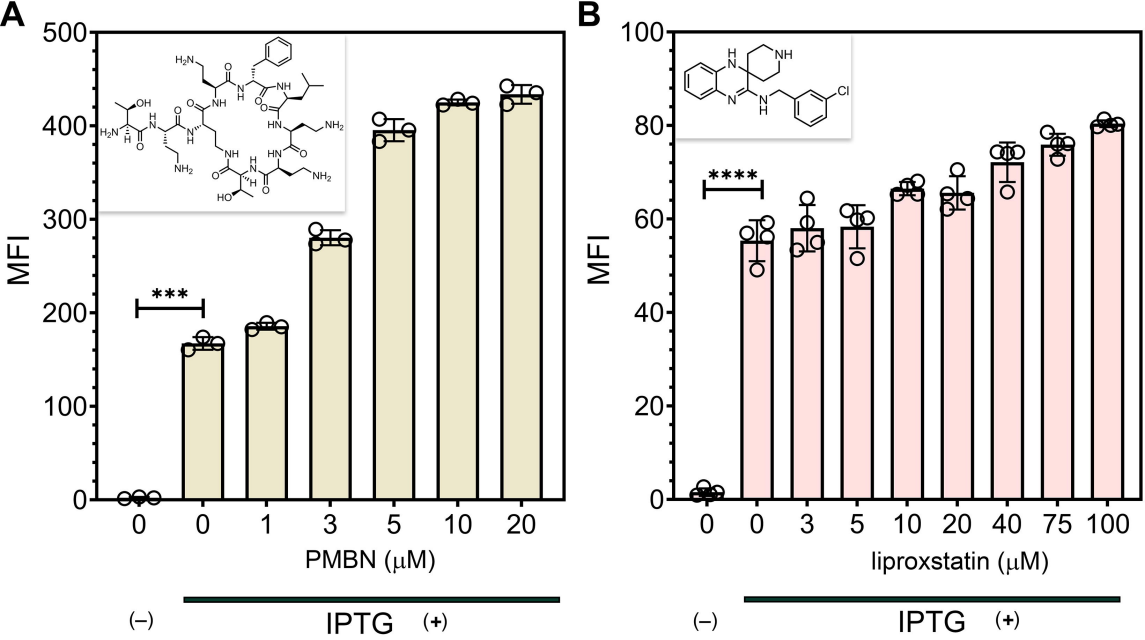
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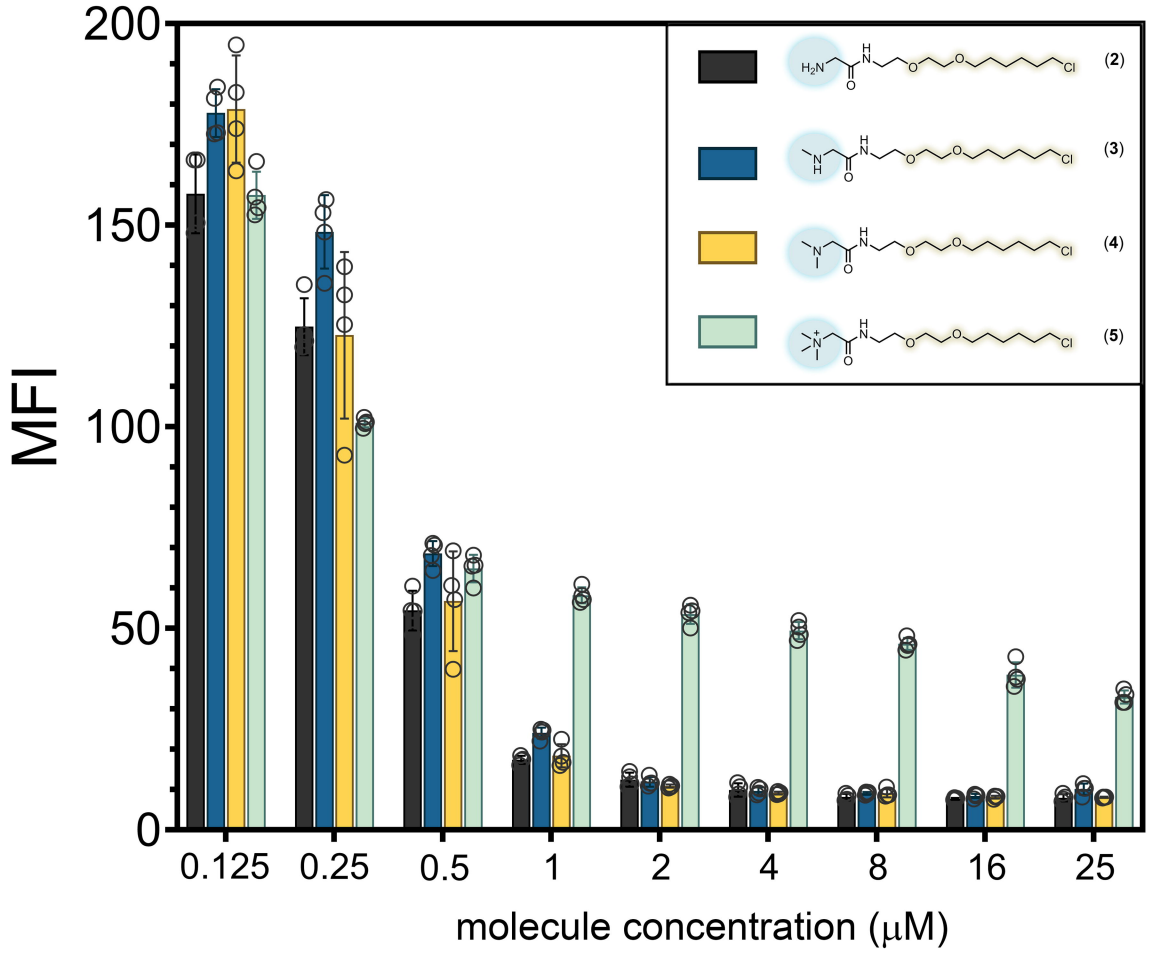
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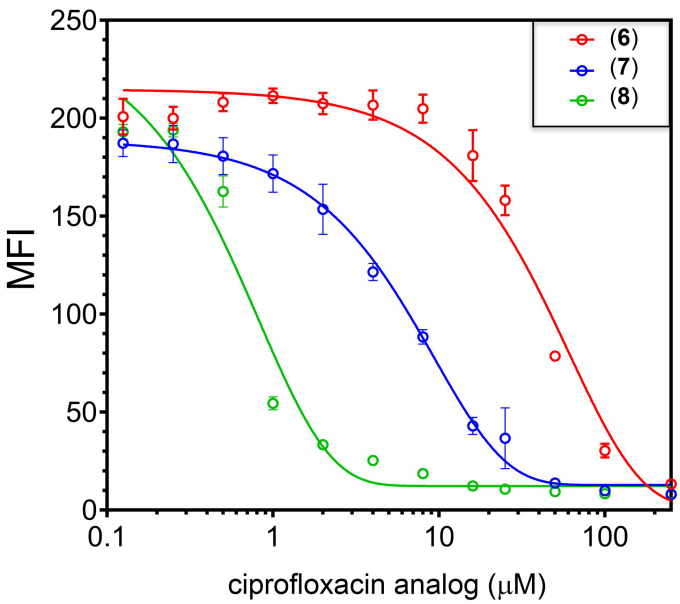
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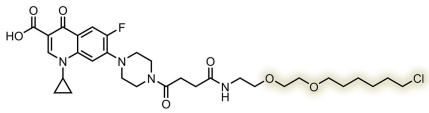
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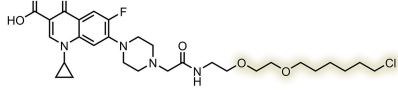
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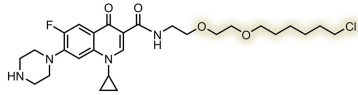
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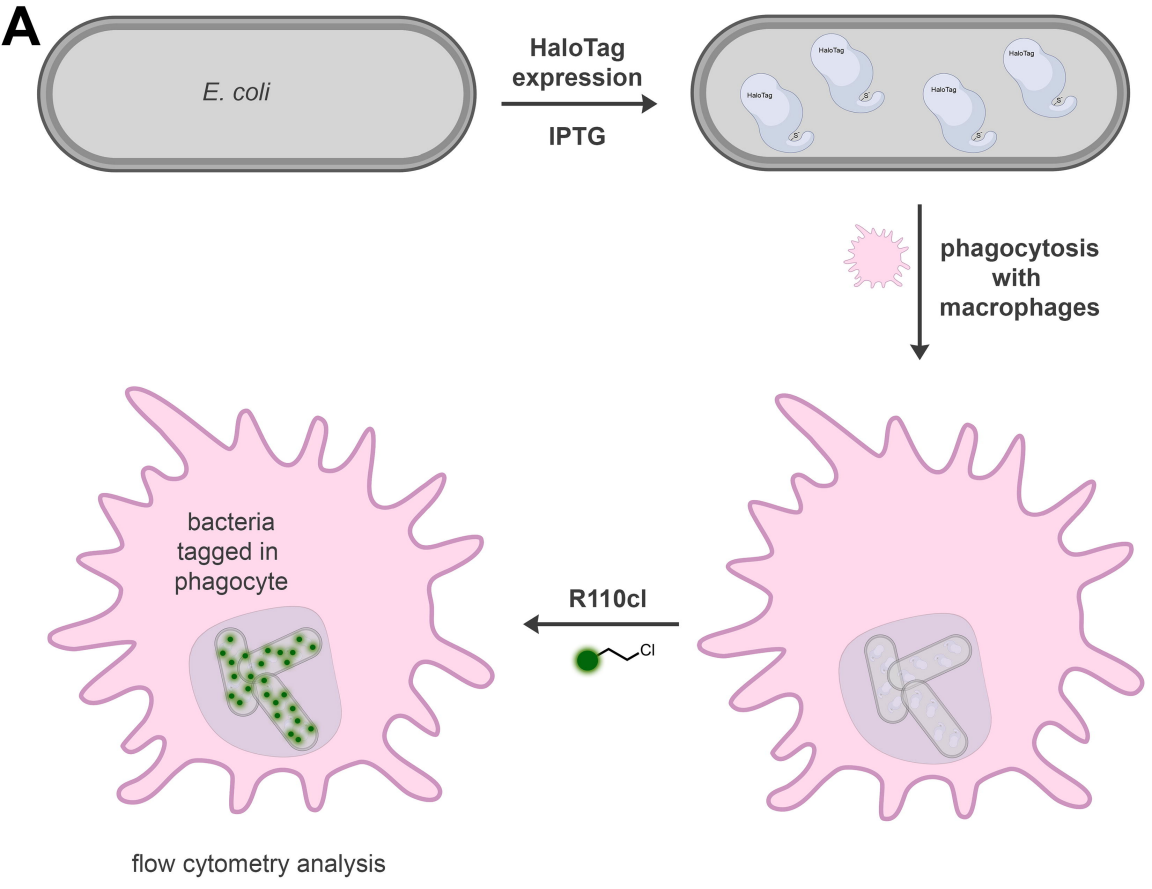
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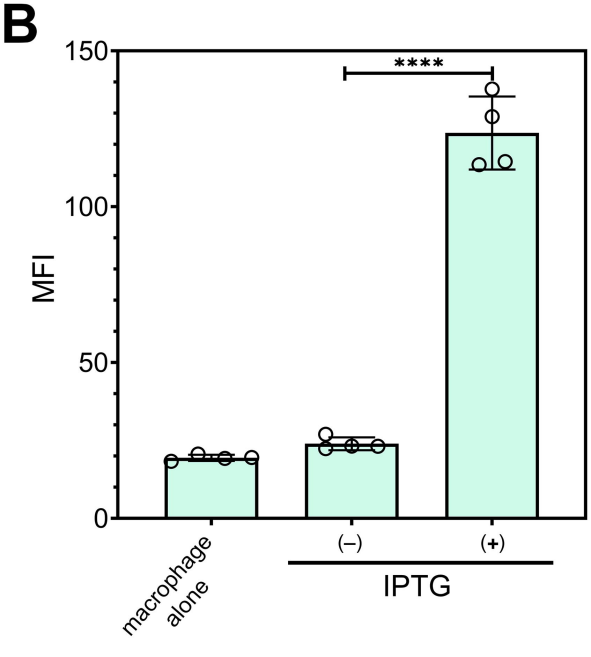
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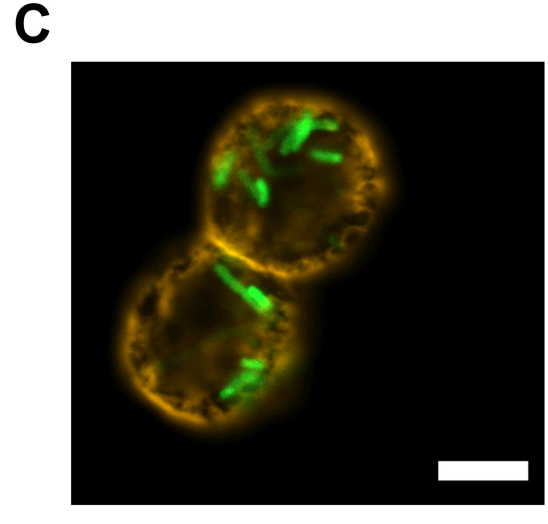
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