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Ribosome-templated azide–alkyne cycloadditions using resistant bacteria as reaction vessels: *in cellulo* click chemistry

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In situ click chemistry, in cellulo click chemistry, macrolide antibiotic, ribosome, solithromycin

ABSTRACT: *In situ* click chemistry has been a powerful method for fragment-based drug design since its discovery in 2002. Recently, we demonstrated that the bacterial ribosome can template the azide–alkyne cycloaddition reaction to expedite the discovery of novel antibiotics. We now report this process can be performed in an antibiotic-resistant bacterial cell. The corresponding triazole products formed *in cellulo* are potent antibiotics that inhibit bacterial growth; moreover, the potency of each cycloadduct can be visualized using the traditional MIC assay in a 96-well plate format. We characterized the *in cellulo* clicked products by independent chemical synthesis and LC–MS analysis, which showed that mass count percent increase was directly proportional to 1/MIC. In other words, potent compounds detected by MIC were formed in greater amounts. Control experiments unambiguously showed the ribosome was responsible for templating triazole formation. Significantly, our method (1) obviates the need to isolate bacterial ribosomes; (2) could be applied to different bacterial strains, which broadens the scope and facilitates the discovery of narrow-spectrum antibiotics; and, (3) does not require the knowledge of mode-of-action and thus could uncover novel antibiotic targets. We believe this method could be expanded and implemented as a novel approach for antibiotic drug discovery.

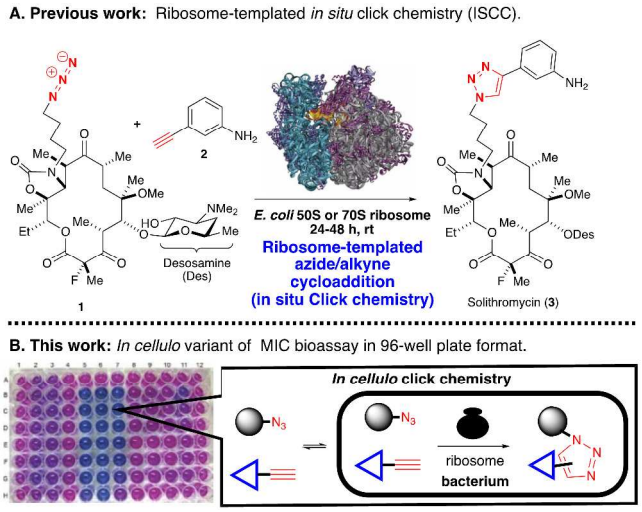
The alarming threat of bacterial resistance over the last few decades has raised significant concerns among those working in the public health sector. The Centers for Disease Control and Prevention reported a rapid increase in the rate of mortality associated with bacteria resistant to antibiotic treatment. Of the two million people diagnosed with severe resistant bacterial infections in the United States every year, approximately 23,000 die as a result of ineffective antibiotic therapy. Since bacteria are naturally subject to spontaneous whole genome mutagenesis and can easily transfer antibiotic resistant genes to other bacteria by means of horizontal gene transfer, antibiotic resistance is unavoidable.[1,2] New antibiotics—and strategies for their efficient discovery and procurement—are desperately needed to tackle this issue.[3-5]

The advent of click chemistry has made a profound impact on fragment-based drug discovery.[6] In 2002, Sharpless and co-workers made a significant contribution when they established the first *in situ* click method wherein the Huisgen [3+2] cycloaddition between azide- and alkyne-functionalized fragments was templated by the enzyme acetylcholinesterase.[7] In this manner, the two fragments that bind in proximity are joined not by Cu(I) but by the biological target (e.g., enzyme), which pays the entropic penalty of bringing the azide and alkyne together to form the triazole product.

Building on pioneering efforts by Sharpless,[8] *in situ* click chemistry has been successfully applied to many other targets including carbonic anhydrase,[9] HIV protease,[10] chitinase,[11,12] histone deacetylase,[13] and DNA.[14] In 2015, Heath employed *in situ* click chemistry to discover in-cell inhibitors of botulinum neurotoxin[15] and found a selective inhibitor of a single point mutation of the Akt1 epitope.[16]

In 2016, we demonstrated that *in situ* click chemistry can be used in the discovery of novel macrolide antibiotics by employing the target—the bacterial ribosome—as a catalyst in the presence of azide- and alkyne-functionalized building blocks. As proof of concept, we showed that best-in-class ketolide solithromycin (**3**) could be prepared *in situ* from azide **1** and 3-ethynylaniline (**2**) (Figure 1A).[17] To date, this is the largest and most complex target to be used to template the Huisgen cycloaddition. Therein, it was shown that the ribosome recognized and directed the precursor subunits in the proper orientation to generate "clicked" triazole products selectively. The clicked products are macrolide inhibitors that target 50S ribosomal subunit. For the *in situ* click method, we correlated the extent of triazole formation with ribosome affinity as revealed by measured K_d values. By comparing the mass count percent increase of each compound in a multicomponent *in situ* click assay, we can screen those compounds rapidly and efficiently.

Figure 1. Ribosome-guided azide–alkyne (A) *in situ* and (B) *in cellulo* click chemistry platforms for drug discovery.



In 2014, Disney and co-workers took the *in situ* click one step further by using the whole cell as a reaction vessel and expressing pathogenic RNA loops to catalyze the synthesis of toxic RNA inhibitors (i.e., *in cellulo* click chemistry) from azide- and alkyne-functionalized fragments.[18] Significantly, this publication verified that the *in situ* click approach could be viable *in cellulo*. A major motivation for Disney's development of *in cellulo* click was the potential solution to a potency versus permeability tradeoff. That is, low molecular weight molecules with weak target affinity are more cell permeable than those with high molecular weight that have stronger target affinity. By having the target *inside the cell* carry out the *in situ* click reaction *in cellulo*, the high molecular weight permeability problem is solved.

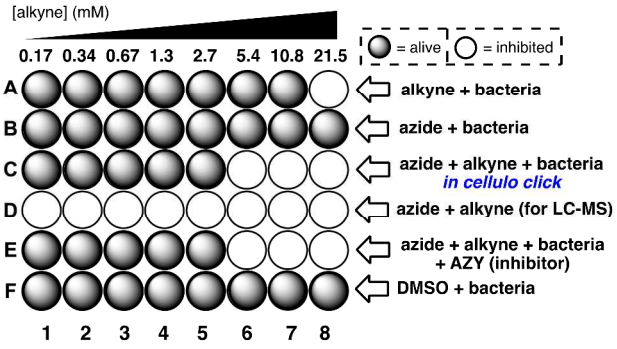
Most recently, Sellstedt elegantly demonstrated that bovine carbonic anhydrase II (bCAII) found in red blood cells could be inhibited *in cellulo*.[19] Significantly, the use of multi reaction monitoring (MRM) mass spectrometry analysis and deuterium-labeled internal standards were key to recapitulating the *in situ* click formation of a powerful bCAII inhibitor first reported by Kolb.[20]

Our motivations for developing *in cellulo* click chemistry (ICCC) were multifold. First, the method obviates the need to isolate bacterial ribosomes, which can be tedious and requires specialized equipment and expertise. Second, the method could be applied to different bacterial strains, which broadens scope and facilitates the discovery of narrow-spectrum antibiotics.[21] Third, the method could be used to selectively target resistant bacterial strains over the wild-type and accordingly expedite the drug discovery process. Based on our *in situ* click method with bacterial ribosomes, we proposed that by employing macrolide-resistant bacteria as reaction vessels, we would leverage the ribosome as a catalyst to synthesize novel antibiotics by combining azide and alkyne fragments without the need to isolate the ribosomes themselves. Once formed *in cellulo*, the antibiotic would inhibit bacterial growth. In addition, the potency of the triazole products could be visually evaluated directly from a 48- or 96-well plate using the well-established MIC assay. The MIC is the lowest concentration of a compound that inhibits bacterial growth.

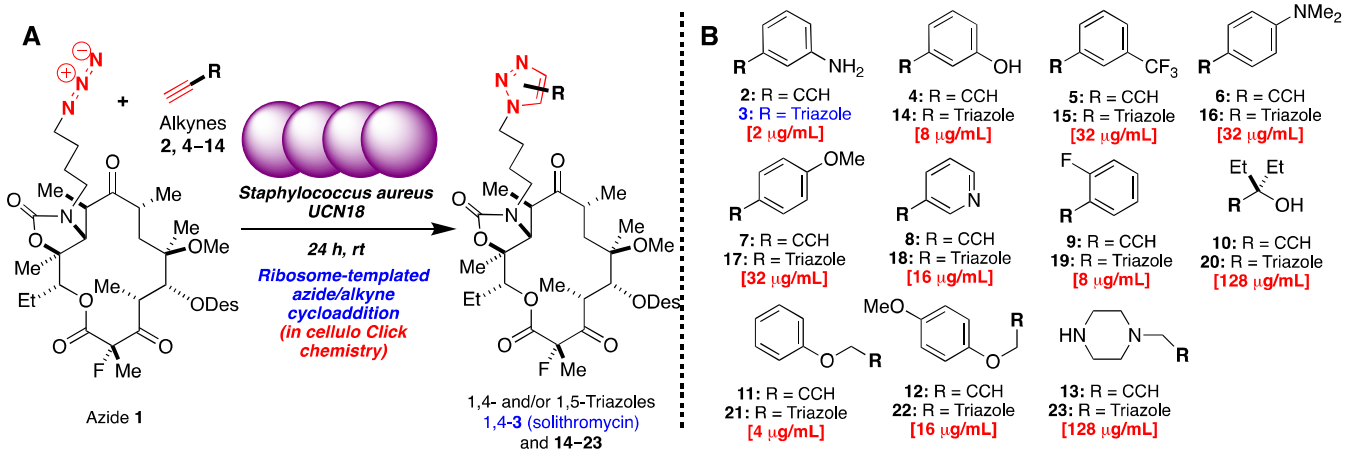
The strain we identified for proof-of-concept studies was the resistant Gram-positive bacterium *Staphylococcus aureus* UCN18, which carries a point mutation at position 2059 (i.e., A2059G). X-ray crystallographic studies of the bacterial ribosome showed that mutation at this specific location conferred resistance towards macrolides.[22] Specifically, ketolides possessing aromatic side-chains such as telithromycin were able to overcome A2059G resistance by making additional contacts within the macrolide binding site. We reasoned that this difference in potency could be leveraged by using *in cellulo* click chemistry (ICCC) wherein the ribosomes in the resistant bacteria template the azide–alkyne cycloaddition and make their own inhibitor. Indeed, the MIC of azide-functionalized macrolide **1** against *S. aureus* UCN18 was 256 μg/mL (in the lag phase) whereas the MIC of solithromycin (**3**), derived from the click reaction of **1** and 3-ethynylaniline (**2**), was 2 μg/mL. In addition, the MIC of 3-ethynylaniline (**2**) was tested and found to be 21.5 mg/mL. The MIC experiments with the individual azide and alkyne fragments also confirmed an important requirement for ICCC; namely, both fragments must be able to enter the bacterial cell by active transport or passive diffusion and engage the ribosome.

Preliminary ICCC experiments were done in a binary fashion utilizing azide-functionalized macrolide **1** and 3-ethynylaniline (**2**) in the presence or absence of bacteria. We hypothesized that wells containing bacteria would template the *in situ* synthesis of solithromycin and result in a lower MIC value whereas those wells lacking the bacteria would not. To test this hypothesis, we carried out *in cellulo* click experiments based on the principle of a MIC assay using 96 well plates (Figure 2). First, a fixed sub-lethal concentration of azide **1** [128 μg/mL] was added to rows B through E whereas only BHI media was added to rows A and F as controls. Second, a stock solution of alkyne (21.5 mM in DMSO) was added to rows A, C, D, and E by two-fold serial dilution beginning with column 8. Third, an overnight culture of *S. aureus* UCN18 was diluted (1:1000 in BHI media) and added to all rows except row D, which was used to quantify the background Huisgen reaction.

Figure 2. Format of *in cellulo* click experiments based on the MIC assay using 48- or 96-well plates.



Scheme 1. (A) Overview of *in cellulo* click experiments. (B) Structures of alkyne fragments in the library and triazoles derived from *in situ* click experiments with MIC values of 1,4-triazoles **3**, **14–23** in square brackets.



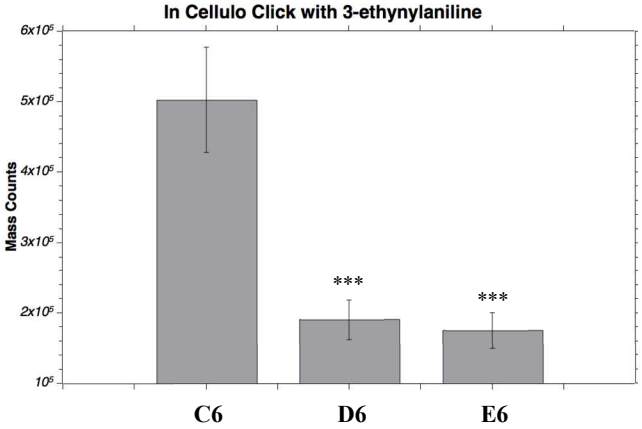
The rationale for dilution was so that bacteria would enter the lag phase wherein the highly active ribosomes of a manageable population of bacterial cells would catalyze the click reaction *in cellulo*. We reasoned that during the exponential phase, bacteria would be dividing at a much higher rate than the competing click reaction, thus complicating the detection of bacterial inhibition. If a potent antibiotic was formed from the click reaction during the lag phase, it would inhibit ribosomal activity and lead to bacterial growth inhibition, which could be detected by the naked eye. Moreover, the triazole (i.e., clicked) product could be detected and quantified by LC–MS.

The results of preliminary experiments are shown in Figure 2 (see SI for details). While 3-ethynylaniline (**2**) has a high MIC, bacterial inhibition was observed at 21.5 mM in well A8. As expected, no bacterial inhibition was observed in row B where a sub-lethal concentration of azide **1** was employed. The *in cellulo* click experiment conducted in row C (i.e., the combination of bacteria, azide, and a concentration gradient of alkyne from lowest in column 1 to highest in column 8) showed that bacterial inhibition occurred in well C6, which was consistent with our hypothesis that the ribosomes are templating the Huisgen cycloaddition *in cellulo*.

To verify that cycloadduct formation by *in cellulo* click chemistry was responsible for the observed MIC difference and not a synergistic effect of the individual fragments, we performed LC−MS analysis (Figure 3).[23] Specifically, we identified solithromycin (**3**) in well C6 by both retention time and mass-to-charge ratio (*m/z*) when compared to an authentic sample prepared by Cu(I)-catalysis.[24] To confirm that solithromycin (**3**) formation was catalyzed by the ribosome *in cellulo* as opposed to another bacterial target or a component in the BHI media (row E), we performed the *in cellulo* click experiment in the presence of ribosomal inhibitor, azithromycin (7.04 mM, see SI for details). By saturating the ribosomes with azithromycin (AZY), binding to azide **1** and the attendant *in cellulo* click reaction are precluded. We recently employed azithromycin in negative control experiments in the development of the ribosome-templated *in situ* click method.[17]

In addition, mass counts (i.e., area) corresponding to solithromycin in these experiments were similar to those obtained from the background Huisgen cycloaddition (row D).

Figure 3. *In cellulo* click and control experiments with 3-ethynylaniline.[a-c]

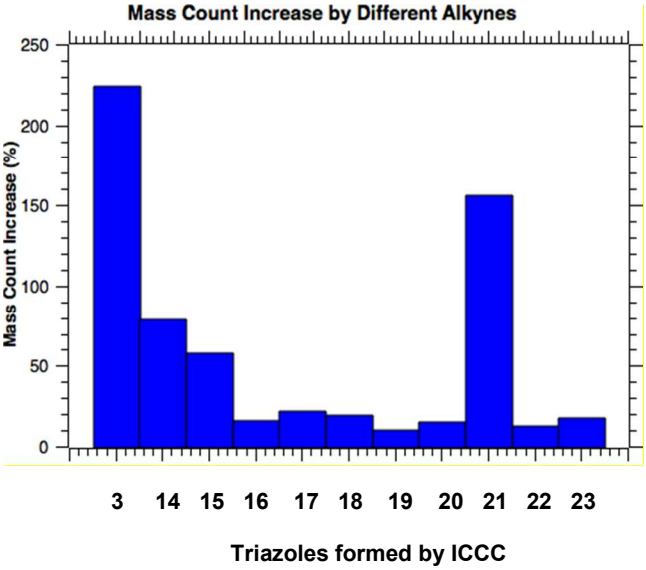


[a] Azide/alkyne/bacteria in well **C6**; [b] Azide/alkyne without bacteria in well **D6**; [c] Azide/alkyne/bacteria/AZY in well **E6**. *** indicates $p < 0.001$ as per two-tailed Student's t-test.

After establishing the viability of *in cellulo* click chemistry (ICCC) for the discovery of novel antibiotics to treat resistant bacterial strains, we selected a library of twelve alkynes to employ our method (Scheme 1). The library featured ten aromatic alkynes, one of which was heteroaromatic (i.e., pyridine **8**), in addition to piperazine **13** and aliphatic alcohol **10**. The experimental protocol followed the 96-well format outlined in Figure 2, and each experiment was run six times for reproducibility (see SI for details). The results are shown in Figure 4. The presence of triazole cycloadducts **3** and **14**–**23** formed *in cellulo* was confirmed with LC–MS by comparison with authentic samples prepared independently by Cu(I) catalysis, which were also used to determine antibacterial activity against *S. aureus* UCN18 in MIC assays (Scheme 1B).[25]

Clicked triazole products displaying greater potency (i.e., antibacterial activity due to ribosome inhibition) will possess lower MIC values. A central premise of *in situ* click chemistry is that targets (i.e., catalysts) favor the formation of more potent inhibitors, which is registered by mass spectrometry as higher mass count (Figure S1).[6-8] Indeed, of the eleven triazoles prepared by ICCC with *S. aureus* UCN18, solithromycin (**3**) with an MIC of 2 μg/mL was formed in the greatest amount. In decreasing amounts, triazoles **21** (4 μg/mL) was formed next, followed by triazole **19** (8 μg/mL) then **14** (8 μg/mL).

Figure 4. Results of ICCC experiments showing mass count percent increases of triazoles **3**, **14**–**23** for the library of eleven alkynes **2**, **4**–**13**.

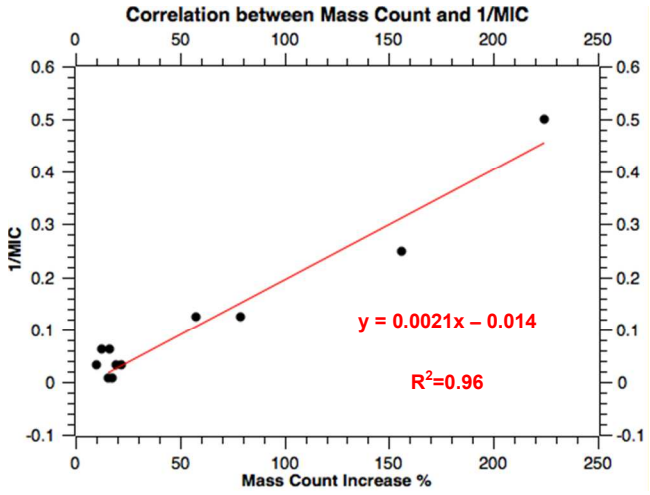


Compounds with greater potency will have a higher reciprocal MIC value (i.e., 1/MIC). Thus, a linear regression plot was generated for mass count percent increase as a function of 1/MIC. The strong correlation ($R^2 = 0.96$) is consistent with trends observed in *in situ* click chemistry (Figure 5).[6-8]

In principle, *in cellulo click chemistry* could be applied in a multicomponent fashion, though our initial trials with multiple alkynes didn't show a strong correlation between MIC and mass count percent increase. We attribute this phenomenon to a marked increase in system complexity wherein differential rates of alkyne diffusion and attendant concentrations result in inconsistent data. The visual observation of bacterial inhibition by ICCC using the MIC platform can arise from three scenarios: (1) additive effects of the two compounds; (2) synergistic effects of the two compounds; or, (3) formation of the clicked triazole. Ultimate confirmation of triazole formation by ICCC comes from LC–MS analysis of the reaction mixture and comparison with an authentic sample to ensure matching retention time and *m/z* values. In addition, the results obtained herein with *in cellulo* synthesis of solithromycin (**3**) match those reported *in situ* click using bacterial ribosomes and ribosomal subunits. In other words, fragments for *in situ* and *in cellulo* click chemistry that possess greater target affinity result in the formation more triazole product.

Beyond the aforementioned advantages of *in cellulo* versus *in situ* click, the isolation of targets from pathogenic strains (for antibacterial drug discovery) is avoided. Furthermore, knowledge of the drug target (i.e., mechanism of action) is not required since any enzyme, in principle, can catalyze triazole formation from the corresponding azide- and alkyne-functionalized fragments. Accordingly, this agnostic approach can be applied to discover new antibiotics with established targets (e.g. ribosome, cell wall, topoisomerase) or more significantly, uncover novel antibiotic targets.

Figure 5. Correlation between MIC and mass count percent increase.



In summary, we have demonstrated that the ribosome-guided *in situ* Huisgen [3+2] cycloaddition (click) chemistry can be performed *in cellulo* wherein bacterial cells serve as reaction vessels. Proof-of-concept studies were performed with the resistant Gram-positive bacterium *Staphylococcus aureus* UCN18, which catalyzed the synthesis solithromycin (**3**) using an azide-functionalized macrolide precursor and 3-ethynylaniline (**2**) *in cellulo*. We extended our method to a library of twelve alkyne fragments and observed a trend consistent with *in situ* click chemistry wherein the most potent compounds as measured by MIC were formed in greatest amount (i.e., mass percent increase). The formation of clicked triazole products was confirmed by performing LC–MS analysis and comparison with authentic material prepared independently by Cu(I) catalysis using both retention time and *m/z* values. We are confident this novel methodology will find utility in the field of antibiotic drug discovery, particularly campaigns focused on addressing the threat of bacterial resistance.

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(25) *In cellulo* click reactions (i.e., *in situ* click chemistry) can result in the formation of two regioisomeric triazoles (i.e., 1,4- and 1,5-cycloadducts), which we observed by LC–MS analysis. Mass counts derived from both regioisomers were combined to calcluate percent increases. See supporting information for details.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and characterization of new compounds. The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

The manuscript was written through contributions of all authors.

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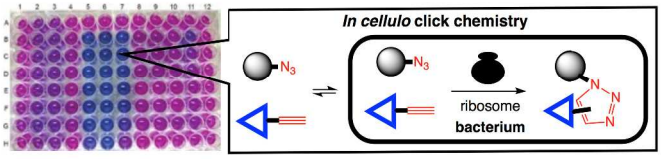
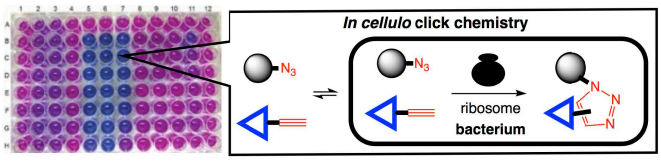
ABBREVIATIONS

ICCC, *in cellulo* click chemistry; ISCC, *in situ* click chemistry; bovine BHI, brain heart infusion; MIC, minimum inhibitory concentration; bCAII, bovine carbonic anhydrase II; MRM, multi reaction monitoring; AZY, azithromycin; DMSO, dimethyl sulfoxide.

Table of Contents (TOC) Graphic:




In cellulo click chemistry
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