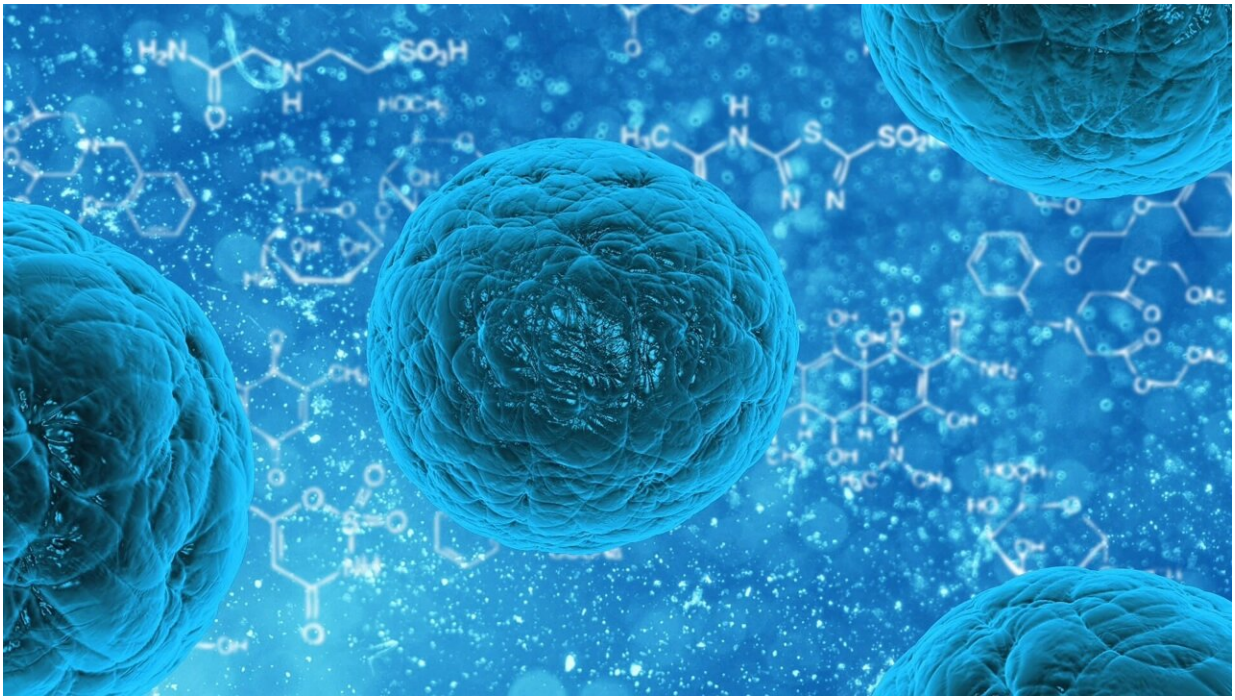
Discovery of a cellular uptake pathway for larger drug molecules

December 8 2022



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Recent findings by UCSF and Arc Institute scientists open up new possibilities for overcoming a fundamental challenge in drug discovery. There can be a tradeoff between optimizing a drug's key-in-a-lock fit with its target and the drug's ability to make its way across the cellular membrane and get to that target in the first place.

Now, a new study published in *Science* from the groups of Kevan Shokat at UCSF and Luke Gilbert at Arc Institute and UCSF reports the discovery of a cellular uptake pathway specifically important for larger drug molecules composed of linked subunits. This knowledge can be harnessed to create new drugs that, although they are large and complex in order to bind optimally to their targets, are efficiently taken up by target cells.

Most traditional pharmaceuticals are small molecules that follow simple molecular rules that promote good cellular uptake and favorable chemical characteristics—including limits on the molecular size and number of sticky chemical groups on the molecule's surface.

However, many key drug targets, such as kinase enzymes often implicated in cancer, are notoriously difficult to target with traditional drugs due to issues with specificity—"there are over 500 human kinase enzymes that are so similar in the pocket where the drug binds, making it a difficult challenge to selectively target a single member of this family and leading to undesirable medication side effects," explains first author of the new study, Kevin Lou.

To mitigate these issues and access new cellular targets that were previously considered "undruggable," some newer classes of drugs have become more large and complex. But there are only a few such examples that step far outside typical chemical rules and retain very effective drug action on intracellular targets.

In general, Gilbert explains that "people have been reluctant to design, synthesize and test such molecules as they are so far beyond standard drug design rules that people just assumed they wouldn't enter cells." Importantly, these larger drug molecules with multiple parts are often much more specific for their targets—they have two molecular keys that have to fit into two adjacent locks simultaneously, boosting specificity.

But it's unclear how these large drugs get into cells, or what design rules we can follow to develop new drugs that take advantage of this molecular linkage to achieve high potency and specificity. By uncovering a cellular gateway allowing these drugs into our cells, the new study "enables scientists to think beyond the standard drug design rules and consider whether larger linked molecules may enable targeting proteins in new ways or targeting proteins we previously thought were untargetable," says Gilbert.

How do you find a new pathway when you don't know what you're looking for, or even whether one exists at all? One powerful way is to conduct genome-wide functional screens that test the importance of individual genes on cellular processes. Several years ago, Gilbert pioneered the development of CRISPRi and CRISPRa screens, in which CRISPR machinery is used with a library of guide RNAs to decrease or increase the expression level, respectively, of one gene at a time across the human genome. When applied to a pool of millions of cells, each cell gets a different modification at a different gene, and researchers can determine which genetic manipulations led to differences in the functional outcome of interest.

In this case, that would mean looking for gene expression manipulations that either made cells more sensitive or more resistant to a linked drug molecule, in comparison to its unlinked counterparts, and tracing that back to determine which genes are important for tuning this process.

Shokat and Gilbert's team conducted these paired CRISPRi (gene repression) and CRISPRa (gene activation) screens in human leukemia cell lines followed by treatment with a large linked experimental anti-cancer drug, RapaLink-1.

It was immediately obvious to the researchers that the linked drug responded distinctly to some of the genetic manipulations as compared

to the response of its component molecules, meaning that RapaLink-1 is dependent on different pathways for cellular entry and/or drug action compared to traditional small drug molecules. Encouragingly, many of the results reinforced each other: repressing a particular gene may cause cellular resistance to the drug, whereas activation of that same gene would promote sensitization to the drug.

The most striking results were a set of three closely related genes that seemed to promote RapaLink-1 activity but had no effect on the unlinked drugs. These genes encode interferon-induced transmembrane (IFITM) proteins, which are known for their roles in antiviral defense, but there has been no prior evidence to suggest that they would impact how drugs work. Just by modulating the levels of the IFITM proteins, the team could drastically change the potency of the RapaLink-1 drug by about 30-fold.

The scientists looked across 659 different cell types and saw a strong correlation between the level of IFITM expression and sensitivity to RapaLink-1, supporting a general role across different types of cells. And expanding to a larger set of 17 different linked and unlinked drug molecules, the researchers determined that the influence of IFITM gene expression was consistent across diverse types of linked drugs.

Previous research has shown that IFITM proteins sit on the surface of our cells, recognize many different types of viruses, and block their entry into our cells—thereby halting infection. But what could they have to do with the cellular response to RapaLink-1? The team measured the ability of cells to slurp up fluorescent versions of RapaLink-1 and related drugs.

"I'll never forget the moment we were first able to visualize that there was less RapaLink-1 uptake in cells with lower IFITM expression," Lou says. The reverse was also true: boosting IFITM levels increased the rate

of drug entry into cells. On the other hand, IFITM protein expression levels had no effect on how well unlinked, "traditional" drugs crossed the cell membrane.

Capitalizing on what they learned, the Shokat lab next designed two new linked drugs that they hypothesized might take advantage of this cellular entry pathway. They generated DasatiLink-1 through a linker-joined combination of two known inhibitors of the leukemia protein BCL-ABL1, known as dasatinib and asciminib. Since each drug binds a distinct pocket on the target protein, the researchers reasoned that the linked version could affix itself to two points of contact like a two-pronged key inserting into two locks, enhancing its specificity and effectiveness.

They also designed BisRoc-1 by linking two molecules of the chemotherapy drug rocaglamide together in a way that would allow it to bridge two copies of the drug's protein target. Remarkably, despite both of these drugs violating traditional drug design principles, the Shokat and Gilbert teams showed that both drugs enter cells, bind tightly to their intended targets, and work just as well as the unlinked versions. The linked versions were uniquely dependent on IFITM protein expression in the target cells, supporting a general role for the IFITM pathway across many types of linked molecules.

Strikingly, the researchers showed that DasatiLink-1 is exquisitely specific for only the BCL-ABL1 kinase, unlike the more relaxed specificity of its two constituent drugs when unlinked. "Linked inhibitors that require a multi-pronged binding mechanism are much more selective," Lou explains, offering substantial advantages as long as they can enter cells efficiently.

Thanks to this study, we now understand a major cellular gateway that linked drugs use to enter human cells. Not only does this answer a long-

standing question in biomedicine, it also paves the way for better molecular linker design that can exploit this entry pathway for higher drug effectiveness and specificity. In the future, Gilbert suggests that it could be possible to "exploit pathways that mediate drug uptake, like IFITM, to boost drug uptake or even to target a drug to select cell types."

Treatments that increase IFITM expression could potentially be given in combination with challenging drugs to boost their cellular entry and get them that much closer to their molecular targets. The discovery of this important pathway expands the frontiers of the molecular features that we can explore in the design of new therapeutic drugs going forward.

More information: Kevin Lou et al, IFITM proteins assist cellular uptake of diverse linked chemotypes, *Science* (2022). DOI: 10.1126/science.abl5829. www.science.org/doi/10.1126/science.abl5829

Provided by Arc Institute