Review

Antibody–Antibiotic Conjugates: A Novel Therapeutic Platform against Bacterial Infections

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Antibodies are potent components of the immune repertoire and have been successfully exploited to treat bacterial infections. Recently an antibody–antibiotic conjugate (AAC) that combines key attributes of an antibody and antibiotic has been shown to be efficacious against *Staphylococcus aureus* infection. An AAC has three components: an antibiotic payload to kill bacteria, an antibody to target delivery of the payload to bacteria, and a linker attaching the payload to the antibody. With increasing understanding of the biology and pathophysiology of *S. aureus*, this article highlights how this knowledge has led to the design principles of an efficacious AAC, and discusses how the AAC platform could be translationally applied to treat other perilous infectious diseases.

Antibodies and Antibiotics as Therapeutics

Approximately two-thirds of antibiotics in clinical use today are natural products or derivatives of natural products. Indeed, antibiotics are Nature’s gifts, having arguably conferred one of the greatest benefits to mankind: their use has reduced mortality and they are crucial for the practice of modern medicine, from invasive surgery to chemotherapy. However, as highlighted at the recent UN General Assembly in New York on 21st September 2016, many common infections are becoming resistant to antibiotics worldwide, resulting in prolonged illnesses and more deaths. At the same time, not enough novel antibiotics or therapeutic modalities are being developed to replace older and increasingly ineffective therapies. At stake are the gains in health and longer lives of the 20th century. Despite our successful exploitation of naturally occurring antibiotics for clinical use, many more-potent antibiotics have not been developed for clinical use because of poor pharmacokinetic properties or toxicities.

Before antibiotics, antibody therapies were the first effective antimicrobials. The discovery by Emil von Behring and Shibasaburo Kitasato that passive transfer of antibodies from the blood of infected animals could provide immunity against diphtheria and tetanus led to the first instance of industrial production of protective serum from sheep for human therapy in 1893. By the early 20th century, serum therapy was used to treat a diverse range of infectious diseases, including pneumococcal pneumonia, meningococcal meningitis, anthrax, and others. Unfortunately, its usefulness was significantly limited by immunological complications, such as serum sickness and immediate hypersensitivity, that were associated with the use of heterologous sera, and was superseded following the discovery of antibiotics. We now know that antibodies are responsible for the observed protection and that they play crucial roles in immune responses. Antibodies protect the host at the earliest time of pathogen encounter through every stage of
pathogenesis, operating through multiple mechanisms. For bacterial infections, the **Fab** (fragment, antigen-binding) region (see Glossary) of an antibody can bind to an antigen and block the interaction of the latter with a cognate ligand, resulting in neutralization of toxins and/or preventing infection. In addition, an antibody can act through its **Fc** (fragment, crystallizable) region to engage the effector arm of the immune system so as to trigger antibody-dependent cellular phagocytosis of opsonized bacteria, and complement-mediated bacteriolysis. Significant advances in the discovery and production of monoclonal antibodies (mAbs) have established mAbs – especially of the immunoglobulin G (IgG) subclass of immunoglobulins – as therapies in oncology, autoimmune disease, and transplant rejection. However, for bacterial infections, while the elicitation of antibody through vaccination has been successful, the use of mAbs as therapeutics (passive immunization) has thus far been met with limited success. Many factors contribute to the limited success of mAbs as a monotherapy to treat bacterial infections. Such factors include the exquisite specificity that mAbs harbor, which, while facilitating precise action, also limits the spectrum of species or strains targeted. Another limiting factor to mAb monotherapy is the concealment of essential epitopes on bacterial surfaces by cell-wall components, such as teichoic acids in the walls of Gram-positive bacteria [1].

A novel therapeutic modality with the potential to treat bacterial infections has emerged that consists of an antibody–antibiotic conjugate (AAC), which combines the key attributes of both antibody and antibiotic in a single molecule. Specifically, the AAC combines the antibacterial effect of an target-specific antibiotic with the superior absorption, distribution, metabolism, and elimination (ADME) properties associated with antibodies, including slow clearance and a long half-life; indeed, a specific AAC has been recently reported to show promise in the treatment of *Staphylococcus aureus* bacteremia [2,3]. We review here how insights into the pathophysiology of invasive *S. aureus* infections were incorporated into the design of an AAC. We also discuss the potential of this AAC as a therapeutic platform to treat bacterial infections and examine the attributes of AAC in conferring pathogen-specificity to broad-spectrum antibiotics. This novel methodology may allow revival of the use of antibiotics previously abandoned because of toxicity or poor pharmacokinetics.

**Clinical Presentation of Persistent *S. aureus* Infection**

*S. aureus* is a leading cause of bacterial infection in humans worldwide and represents a major health problem in both hospital and community settings. Recent multicenter studies in the USA have revealed that, among hospital-acquired infections (HAIs) requiring antibiotic treatment, *S. aureus* was the second most frequently reported causative pathogen following *Clostridium difficile* [4], and the most common pathogen among hospitalized patients with ventilator-associated pneumonia [5]. Moreover, the prevalence of healthcare-associated *S. aureus* endocarditis in industrialized regions has increased over the past decade from approximately 10% to 25% [6].

Infection with *S. aureus* has become increasingly difficult to treat owing to the emergence and rapid spread of methicillin-resistant *S. aureus* (MRSA) strains, increasing resistance and dose-limiting adverse events with current antibiotics such as vancomycin [7]. For *S. aureus* bacteremia, failure of antibiotic treatments without measurable outgrowth of antibiotic-resistant strains is not uncommon [8]. Survival of these antibiotic-susceptible subpopulations of *S. aureus* is likely due to multiple factors, including reduction or cessation of growth – as observed in infectious endocarditis – as well as heteroresistance and survival inside host cells. There is growing evidence that *S. aureus* can survive and persist in host cells (Box 1). Intracellular persistence inside host cells allows *S. aureus* bacteria to escape from antibiotics. Indeed, much higher concentrations of antibiotics, often above the concentrations achievable in serum, are required to kill intracellular *S. aureus* than to inhibit the growth of planktonic
The response is a highly conserved stress response that is triggered by a variety of stresses, including toxins and other environmental insults [16]. The SCV phenotype has been proposed as a strategy for intracellular persistence because of studies using antibiotics and other environmental insults. This antibiotic tolerance is transient and reverts to genetically identical, metabolically slow-growing cells that can survive lethal concentrations of antibiotics [17]. SCVs is given in [17]. Prosthetic joint infections [19] are central to the formation of persisters. The stringent response is a highly conserved stress response that is triggered by the stringent response

**Physiological States of Intracellular S. aureus**

The precise physiological state(s) of intracellular S. aureus remain to be fully characterized and are likely to be heterogeneous. The intracellular environment has been shown to have a greater capacity to induce the formation of small colony variants (SCVs) [13] and persister cells [14]. First described more than 100 years ago for S. aureus, SCVs are characterized by common phenotypic features such as slow growth, altered susceptibility to antibiotic agents [15], and are frequently auxotrophs. SCV auxotrophs are primarily due to either deficiencies in electron transport – as shown for menadione- and/or hemin-auxotrophs – or deficiency in thymidylate biosynthesis, all of which may be a consequence of downregulation of citric acid cycle activity [16]. The SCV phenotype has been proposed as a strategy for intracellular persistence because it is a pathogenic form that facilitates persistent and recurrent S. aureus infections [17]. SCVs have been isolated from patients with persistent infections, such as in cystic fibrosis [18] and prosthetic joint infections [19]. A recent review on the clinical significance of staphylococcal SCVs is given in [17].

By contrast, persisters, first described for S. aureus in 1944 [20], are a subpopulation of genetically identical, metabolically slow-growing cells that can survive lethal concentrations of antibiotics and other environmental insults. This antibiotic tolerance is transient and reverts to wild-type with a new population of persisters [21]. Persisters can emerge stochastically or be environmentally induced. Our mechanistic understanding of persistence is derived primarily from studies using Escherichia coli as the model organism. In E. coli, the stringent response and toxin-antitoxin (TA) systems are central to the formation of persisters. The stringent response is a highly conserved stress response that is triggered by a variety of stresses,
including nutrient deprivation, and is mediated by guanosine tetraphosphate and pentaphosphate, (pppGpp) [22]. Once produced, these alarmones or secondary messengers function as a cellular switch to shut down replication, transcription, and translation, and induce genes involved in the stress response [23]. The TA system consists of two components, a stable toxin that inhibits cell growth, and an antitoxin that regulates toxin activity. High levels of (pppGpp) induce a cascade of molecular events, including the stimulation of Lon protease to degrade the antitoxin, leaving free and activated toxins to inhibit translation and cell growth, thereby promoting the persistence state [24]. A subpopulation of Salmonella that is internalized by macrophages has also been shown to form persisters by the (pppGpp-) and TA-dependent mechanism [14]. Although the mechanistic understanding of S. aureus persister formation is less clear, (pppGpp) has been shown to bind and inactivate GTPase to dampen cell proliferation at a post-transcriptional level by actively interfering with ribosome assembly to inhibit cell growth, leading to increased antibiotic tolerance – a mechanism that appears to be conserved in Gram-positive bacteria [25]. Although S. aureus harbors at least three annotated TA systems, their role in persister formation could not be confirmed because knockout of all TAs had no effect on the level of persisters in exponentially growing or stationary-phase cells [26]. Instead, formation of S. aureus persisters was associated with stochastic entry into stationary phase and an accompanying drop in intracellular ATP [26]. Whether this is a general mechanism of tolerance that also governs persister formation in other bacteria remains to be determined. Regardless of the variation in molecular mechanisms, the persister state has been found for a variety of pathogens, including S. aureus, Pseudomonas aeruginosa, and mycobacteria, and is implicated in the recalcitrance of chronic infectious disease to antimicrobial therapy, as in the cases of cystic fibrosis and tuberculosis [27–29].

Intracellular S. aureus contributes to persistent and hard-to-treat infections: by residing within host cells the bacteria are protected from attack by extracellular host defenses; and, by adopting the semi-dormant state, they become intrinsically resistant to antibiotic therapy [30–33]. The AAC therapeutic provides the possibility to specifically tag and kill S. aureus because the bacteria periodically escape from their intracellular niches. When AAC-tagged bacteria enter cells that already contain resident S. aureus, the delivery of a high concentration of antibiotic into the intracellular compartment can eliminate tagged, as well as untagged, resident bacteria that are not adequately eliminated by traditional small-molecule antibiotics (2,3]; see AAC Mechanism of Action).

Design of an AAC

The AAC is a variant of the antibody–drug conjugate (ADC) concept that has been applied successfully to cancer treatment. Whereas ADC uses mAbs to selectively deliver potent cytotoxic payloads to antigen-expressing tumor cells [34], the AAC uses the antibody to deliver an antibiotic payload to bacteria. The AAC consists of three building blocks: antibiotic payload to kill bacteria, an antibody to target the delivery of the payload to the bacteria, and a linker to attach the payload to the antibody and allow its release once the AAC is internalized by mammalian cells. For therapeutic applications, the AAC must satisfy demanding criteria concerning specificity, safety, and stability to achieve effective delivery of the payload to the target. All three components – antibody, linker, and payload – play crucial roles in defining target specificity, degree of stability, and mechanism of action, respectively. In the following section we describe the design of one specific AAC, a THIOMAB™ antibody–antibiotic conjugate that is currently being investigated as a potential therapeutic against hard-to-treat S. aureus infections (Figure 1).

The Antibody
The β-N-acetylgalcosamine cell-wall teichoic acid (β-GlcNAc-WTA) antibody is one of many human mAbs of the IgG1 subtype isolated from S. aureus-infected patients.
The β-GlcNAc-WTA mAb binds specifically to β-GlcNAc residues on the ribitol phosphate units of wall teichoic acid (WTA, Figure 2). This antibody was selected for AAC because it fulfilled several criteria. For example, the antigen to which the antibody binds is highly abundant and highly expressed on S. aureus in vitro and during infection, and is absent from mammalian cells [2,35]. Moreover, it was estimated that about 50 000 β-GlcNAc-WTA antibody-binding sites are present on a single S. aureus bacterium [2]. Lastly, the TarS glycosyltransferase responsible for appending β-GlcNAc onto ribitol phosphate is conserved in all MRSA [36], and is crucial in conferring resistance to β-lactam antibiotics and other acting enzymes acting on the cell wall [37].

Common methods of conjugating payloads to mAbs include alkylation of reduced interchain disulfides, acylation of lysines, and alkylation of genetically engineered cysteines. There are eight interchain cysteines and up to 100 lysines available for conjugation on IgG1 mAbs, and therefore conjugation to these sites might result in heterogeneous products containing a mixture of species with different molar ratios of drug to antibody linked at different sites [38]. Lessons learned from the design and clinical development of ADCs has indicated that the level and placement of conjugated drugs could impact not only on pharmacokinetics but also on the activity, potency, and tolerability of the bioconjugate [39]. The stoichiometry of 2–4 drugs per mAb appears to be optimal because more heavily loaded conjugates are cleared very rapidly from the circulation [39]. For the S. aureus AAC, the THIOMAB™ technology was adopted to resolve the issue of conjugate heterogeneity by directing the attachment of drugs to defined sites, and with near-uniform stoichiometry [40]. Specifically, the valine 205 position of the β-GlcNAc-WTA antibody light chain was substituted with a reactive cysteine to allow drugs to be conjugated with defined stoichiometry without disruption of interchain disulfide bonds. It has also been shown that attachment to this position on the mAb does not interfere with the antigen-binding and Fc functions of the antibody [40] and generates a linkage that is stable in mouse plasma [41]. Moreover, a high yield and highly homogeneous AAC, with a drug–antibiotic ratio (DAR) of 1.9, was achieved with this approach [2].

The Antibiotic

Engagement of the β-GlcNAc-WTA antibody to its antigen does not significantly impact on S. aureus virulence in a mouse bacteremia model [2]. In this infection model, a MRSA strain was inoculated through the mouse tail vein. Treatment with either β-GlcNAc-WTA antibody or the isotype control antibody was initiated 24 h post-infection. Enumeration of bacterial load in the kidney at day 4 post-infection showed no statistical difference between β-GlcNAc-WTA antibody and control [2]. Thus, the antibacterial effect must be determined by the antibody. An optimal antibody for AAC must fulfill a set of criteria. First, as detailed in the linker section below, the antibody must possess a functional group that is amenable to conjugation. If a functional group is absent, modifications to introduce a functional group to the antibody to enable conjugation must not result in loss of antibacterial potency. Second, because intracellular S. aureus are primarily in the dormant state, the antibiotic must be active against bacteria in the stationary phase or persistor state, or, alternatively, consist of SCVs. Third, the antibody must also retain bactericidal activity in acidic pH of the lysosomal environment and exhibit long retention time within mammalian cells upon release as a free drug. Finally, it must be highly potent because its delivery is limited by the antigen copy number at the surface of the bacteria. Some of these properties are illustrated by the comparison between two members of the ansamycin class of antibiotics, rifampicin (also known as rifampin) and dimethyl DNA31 (4-dimethylamino piperidino-hydroxybenzoxazinorfamycin, a rifalazil analog, referred to as rifalog in [2], and hereafter referred to as dmDNA31) that inhibit bacterial RNA polymerase (Tables S1 and S2 in the supplemental information online). The two antibiotics have comparable potency as measured by the standard minimal inhibitory concentration (MIC) assay at neutral and acidic pH. When conjugated to the β-GlcNAc-WTA antibody by the MC-ValCit-PABQ linker Long half-life: the half-life of a drug is the time it takes for half of a given dose to be eliminated from the body or bloodstream. Antibody–drug conjugates typically have low clearance and long half-lives similar to the parent antibody. MC-ValCit-PABQ linker: consists of maleimide and caproic acid (MC) for the attachment to an antibody, valine citrulline (ValCit) as the protease-cleavable dipeptide, and a novel p-aminobenzyl quaternary ammonium salt (PABQ) for attachment to dmDNA31. Minimum inhibitory concentration (MIC) assay: lowest concentration of a chemical (antibiotic) that prevents the visible growth of a bacterium. Mouse bacteremia model: in this case, S. aureus infections carried out by intravenous injection into the tail vein of female BALB/c mice aged 7 weeks. Osporized bacteria: a pathogen that is marked by a process of opsonization for ingestion and elimination by a host phagocytic cell. This involves binding of an opsonin, for example antibody, to an epitope on an antigen. The Fab portion of the antibody binds to the antigen, whereas the Fc portion binds to the Fc receptor on the phagocyte, facilitating phagocytosis. Osporophagocytosis: phagocytosis initiated by opsonins. These are molecules (e.g., complement, antibodies) that mark antigens (e.g., bacteria) and enhance their uptake by host myeloid cells for further destruction and elimination. Persisters: a subpopulation of bacteria capable of growing after antibiotic killing. This new population can be killed again by the same antibiotic except for a small residual population. Residual organisms are not antibiotic-resistant mutants but are instead dormant cells that have entered a non- or extremely slow-growing physiological state rendering them insensitive (refractory or tolerant) to antimicrobial treatment. Phagosome: vesicle formed around a particle absorbed by phagocytosis by fusion of the cell membrane around the particle. It is a compartment in which pathogenic organisms can be killed and digested. Phagolysosome: cytoplasmic body formed by the fusion of a
dmDNA31 was active as an AAC. The activity of dmDNA31-AAC reflects the properties of dmDNA31 that are either absent or weak in rifampicin: dmDNA31 has potent bactericidal activity against persisters and stationary-phase S. aureus, and is well retained in macrophages [2]. Thus, at least for S. aureus infections, the antibiotic must be able to kill dormant bacteria and harbor prolonged cellular retention.

The Linker

An important feature of the linker is that it must avoid nonspecific release while in circulation but can be rapidly degraded to release the payload following internalization and processing in the endosomal or lysosomal pathway. Stability of the drug-linker in circulation ensures long circulating half-life of the AAC and provides exposure for several days post-injection to capture bacteria that have escaped from lysed host cells [3]. Key components of a cleavable linker are (i) a reactive group for covalent linkage to the targeting antibody (attachment group), (ii) a conditionally stable release mechanism, and (iii) a self-immolative spacer for attachment to the antibiotic payload.

For the S. aureus AAC, the MC-ValCit-PABQ linker was selected for covalent attachment of the β-GlcNAc-WTA antibody to dmDNA31 (Figure 1 and Box 2). The MC-ValCit-PABQ linker consists of maleimide and caproic acid (MC) for attachment to the antibody, valine citrulline (ValCit) as the protease-cleavable dipeptide, and a novel p-aminobenzyl quartenary ammonium phagosome with a lysosome that contains hydrolytic enzymes.

**Box 2. Linker Chemistry**

Several conditionally stable release mechanisms that avoid systemic release are available. These include hydrazones, disulfides, glucuronides, and peptides. Hydrazones are relatively stable at neutral pH in the bloodstream (pH 7.3–7.5) but are hydrolyzed within the acidic environment of target cell endosomes (pH 5.0–6.5) and lysosomes (pH 4.5–5.0) to release an active drug. Disulfides release a drug upon reduction by intracellular thiols. Cleavable peptide and glucuronide linkers that are specifically hydrolyzed by intracellular enzymes are emerging as the release mechanism of choice because they are generally more stable in circulation than disulfides and hydrazones [71,72].

For the Staphylococcus aureus AAC, the MC-ValCit-PABQ linker was selected. Most ValCit linkers utilize p-aminobenzy carbamate (PABC) as a spacer for attachment to the bioactive payload. PABC hydrolytically decomposes upon deacylation to spontaneously release free active drug upon cathepsin cleavage. The clinically approved ADC Adconitris (brentuximab vedotin), where monomethyl auristatin E (MMAE) – a potent microtubule inhibitor linked to a CD30 antibody – has been successfully used for the treatment of Hodgkin’s lymphoma and anaplastic large-cell lymphoma.

Although this carbamate-based linkage chemistry is utilized in approximately half of the ADCs currently in clinical trials [34], PABC has several drawbacks. First, it requires that the bioactive payload contain either a primary or secondary amine functional group to form a carbamate or amide linkage [73]. Many antibiotics, however, do not contain such reactive functional groups that are amenable to this reversible modification. Instead, RNA polymerase inhibitors, such as dmDNA31, and rifabutin, as well as the protein synthesis inhibitors retapamulin and clindamycin, contain tertiary- or heteroaryl-amine functional groups. One approach to enable carbamate-linkage chemistry for molecules containing the tertiary amine functional group is exemplified by the natural product auristatin E and its analog MMAE. The tertiary amine of auristatin E has been modified to a secondary amine by the removal of a methyl group of auristatin E, resulting in MMAE with similar potency [46]. This is not a viable strategy if the tertiary amine is necessary for activity, or is part of a structural element, because such a modification could result in significant loss in potency or conjugate activity [46].

Second, most antibiotics are somewhat hydrophobic, and hydrophobicity of the linker-drug is positively correlated with the propensity for aggregation of the antibody upon attachment of the linker-drug [46,74]. Attaching an antibiotic containing a secondary amine to a hydrophobic linker and replacing the charged amine with a carbamate would have the undesirable effect of increasing hydrophobicity of the linker-antibiotic conjugate for attachment to the antibody.

A potential solution to these drawbacks is a novel self-immolative p-aminobenzyl quartenary ammonium salt (PABQ) spacer that can be attached to the tertiary or heteroaryl amines (see Figure 1 in main text). Cathepsin cleavage of the amide at the C terminus of citrulline followed by self-immolation of PABQ results in the release of active dmDNA31, as shown in murine peritoneal macrophages [2,46].
salt (PABQ) for attachment to dmDNA31. Linkage between MC and the antibody occurs through the thiol-maleimide chemistry that couples the reactive thiol in the engineered cysteine of the antibody to maleimide \[42\].

The ValCit dipeptide was selected because it has been shown to offer maximum serum stability and is cleaved by multiple lysosomal cathepsins including cathepsins B and L \[43,44\]. These cysteine proteases are highly and ubiquitously expressed but restricted to the endo- and phagolysosome compartments of various tissues and are conserved across species \[45\]; they are predominantly active and stable at slightly acidic pH such as found in the endolysosomal vesicles, but are relatively unstable at neutral pH, such as that of the extracellular milieu.

The MC-ValCit-PABQ linker was shown to be highly stable in plasma as well as under physiologic conditions in mice, and was efficiently cleaved to release active payload within minutes of internalization into mammalian cells, such as in murine peritoneal macrophages \[2,3,46\]. The utility of PABQ-mediated linkage is not limited to the ValCit linker and has also been shown to be stable in mouse and rat plasma, and in circulation in rats when combined with a glucuronide linker \[47\].

Comparison between a carbamate-linked (DNA31-AAC) and a quarternary-ammonium-linked AAC (dmDNA31-AAC, Figure 3) has uncovered an unexpected impact of amine substitution on AAC activity, independent of antibiotic potency. DNA31 differs from dmDNA31 only by the absence of the two methyl groups on the amine distal to the binding site of the rifamycin core to RNA polymerase, but is otherwise equipotent as an antibiotic \[46\]. However, dmDNA31-AAC is more efficacious than DNA31-AAC at reducing intracellular S. aureus load in macrophages, an effect that is associated with an optimal placement of lipophilicity and charge of the released
In the future, as linker technology improves, it is likely that more payloads with different functional groups could be accommodated. Recently, a ValCit linker that allows for payload attachment at an aliphatic alcohol through phosphate bridging has been reported [48]. These aqueous soluble phosphate-containing drug-linkers were found to have robust plasma stability coupled to rapid release of payload in lysosomal lysates from rat liver cells [48].

Figure 2. Structure of Staphylococcus aureus Wall Teichoic Acid (WTA) and the Epitope of the Anti-\(\beta\)-GlcNAc-WTA mAb. WTAs are anionic glycopolymers that are covalently attached to peptidoglycan via phosphodiester linkage to the C6 hydroxyl of the N-acetyl muramic acid sugars. The precise chemical structures of WTAs are highly diverse among Gram-positive bacteria. (A) In S. aureus, WTA is a polymer of ribitol phosphate (yellow) covalently linked to the 6-OH group of N-acetylmuramic acid residues in peptidoglycan via a disaccharide consisting of GlcNAc-1-P and N-acetylmannosamine (blue and purple, respectively) followed by two glycerol-phosphate units (orange). (B) The glycerol-phosphate and ribitol phosphate units can be modified by the addition of cationic D-alanine mediated by dltABCD operon enzymes. In addition to anlylation, the phosphoribitol repeating units are also appended with either \(\alpha\)- or \(\beta\)-O-linked N-acetylgalactosamine (\(\alpha\)-GlcNAc or \(\beta\)-GlcNAc) by the action of TarM and TarS glycosyltransferases, respectively [36]. The \(\beta\)-GlcNAc-WTA mAb of the antibody–antibiotic conjugate (AAC) binds specifically to the \(\beta\)-O-linked N-acetylgalactosamine moieties of the WTA.

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Figure 3. Carbamate Linkage to DNA31 and Quaternary-Ammonium-Linked dmDNA31. Chemical structures are shown for a maleimide caproic acid (MC)-containing ValCit linker linked to DNA31 through a carbamate (A), and to dmDNA31 through a quaternary-ammonium salt (B).
In Vivo Stability and Pharmacokinetics of the \textit{S. aureus} AAC

For ADCs, it is well known that, despite best efforts to design linkers to be entirely stable in plasma, unanticipated chemical or enzymatic activity \textit{in vivo} could lead to breakdown, chemical modification, or deconjugation of the ADC \cite{38,49}. It is therefore important that, as with ADCs, the integrity and pharmacokinetics of AAC molecules are assessed \textit{in vivo}. Using multiple bioanalytical methodologies, plasma concentrations of total antibody, antibody-conjugated dmDNA31, and unconjugated dmDNA31 were measured following intravenous administration of a single dose of AAC ranging from 5 to 50 mg/kg in non-infected and \textit{S. aureus}-infected mice \cite{3}. At the tested dose-range, the AAC molecules showed similar \textit{in vivo} profiles to the unconjugated mAb: a short distribution phase, a long elimination phase, and a long half-life. The AAC molecules also presented systemic exposures that were dose-proportional, and similar pharmacokinetic behavior between non-infected and infected mice was observed \cite{3}. Importantly, unconjugated dmDNA31 plasma concentrations were very low, indicating \textit{in vivo} stability of the linker, and minimal antibiotic deconjugation while the AAC molecules were in circulation \cite{3}. Moreover, AAC clearance in infected mice compared to non-infected mice was only marginally increased, and this might have resulted from the expected increase in deconjugation associated with uptake of the AAC–bacteria complexes \cite{3}. This study indicated that AAC molecules were largely stable as a prodrug in the circulation in mice, undergoing deconjugation to release free drugs only when the AAC–bacteria complexes are processed within phagolysosomes \cite{3}. Whether these properties will continue to hold true for \textit{S. aureus}-infected patients will await the outcome of clinical trials.

\textbf{AAC Mechanism of Action and In Vivo Efficacy}

The mechanism of action of AAC comprises internalization of extracellular \textit{S. aureus} and killing of intracelluar \textit{S. aureus} bacteria by intracellular release of the active antibiotic (Figure 4). While in circulation, the AAC is a prodrug because the covalently linked dmDNA31 is inactive \cite{2}. The high affinity of the \(\beta\)-GlcNAc-WTA antibody to the highly abundant antigen on the surface of \textit{S. aureus} ensures that \textit{S. aureus} circulating in the bloodstream, or released from lysed infected host cells, are rapidly opsonized or tagged with TACs; a single bacterium can be coated with as many as 50 000 AAC molecules \cite{2}. The \textit{S. aureus}–AAC complexes are then internalized by both phagocytic and non-phagocytic cells \cite{2}. For phagocytic cells, such as neutrophils and macrophages, opsonized (AAC-tagged) bacteria are internalized through opsonophagocytosis. For non-phagocytic epithelial and endothelial cells, entry of AAC-tagged bacteria occurs through the endogenous host invasion mechanism of \textit{S. aureus}, such as that mediated by fibronectin-binding protein \cite{2,50}. When AAC-tagged bacteria are sequestered within acidic endo- or phagolysosomes, cathepsins released within these subcellular vesicles can cleave the ValCit linker and release active antibiotics. Entry of AAC-tagged bacteria into cells containing resident \textit{S. aureus} and subsequent intracellular release of active antibiotic in these cells can then result in the elimination of tagged and bystander killing of untagged resident bacteria. By this mechanism, AAC molecules are able to concentrate active dmDNA31 specifically in a location where \textit{S. aureus} is poorly treated by conventional antibiotics. Indeed, \textit{in vitro}, AAC-tagged \textit{S. aureus} have been shown to be efficiently killed inside every cell type tested, including human macrophages as well as human endothelial and epithelial cell lines \cite{2}.

As noted in Box 1, a significant proportion of \textit{S. aureus} in a natural infection reside within host cells at some point during the course of infection, and therefore the time spent inside host cells might provide a significant opportunity for the bacterium to evade antibiotic activity. This is demonstrated in a mouse bacteremia model where, although vancomycin was highly efficacious when administered within 1 h after the initiation of infection, efficacy of the antibiotic was limited when administered 24 h post-infection \cite{2}; poor efficacy of vancomycin following delayed treatment might be explained by intracellular bacteria being shielded from antibiotics. By contrast, because the AAC was able to kill intracellular bacteria as well as tag bacteria that...
periodically escape from lysed cells for internalization and intracellular killing, administration of a single dose of AAC at 24 h post-infection could reduce the presence of *S. aureus* in the kidneys at day 4 post-infection (to the limit of detection), and was found to be superior to the clinically equivalent, twice-daily dosing of vancomycin [2].

Structurally, the antibody component of an AAC accounts for the majority of the therapeutic agent (approximately 98% of total AAC by molecular weight). Biologically, the underlying antibody backbone confers properties such as target-specific binding, neonatal receptor (FcRn)-dependent recycling, and Fc effector functions that strongly influence the pharmacokinetics of AACs. Therefore, linking the antibiotic to an antibody could significantly improve the half-life of antibiotics *in vivo*. For example, the half-life of dmDNA31 as a free drug in mice could be extended from 3–4 h to approximately 4 days when conjugated to the β-GlcNAc-WTA...
antibody [3]. Consequently, the improved pharmacokinetics of AAC enable the delivery of sufficient antibiotic to cellular regions where *S. aureus* is concentrated, while limiting the overall dose of antibiotic that needs to be administered systemically. Moreover, it has been shown that a single dose of AAC can substantially reduce bacterial load in the kidneys, heart, and bones of *S. aureus*-infected mice at 7 and 14 days post-dosing, implying that the AAC can be adequately distributed throughout these tissues [3]. At the efficacious dose, infection by *S. aureus* presented minimal effects on the pharmacokinetics of the AAC, suggesting that target-mediated clearance might play a minor role – at best – in clearing the AAC [3].

**Potential Utility and Challenges of AACs as a Therapeutic Platform for Infectious Diseases**

Insights from the design and development of ADCs and the *S. aureus* AAC have provided optimal characteristics of the antibiotic, antibody (and antigen), and linker for an AAC (Figure 5, Key Figure). The AAC as a therapeutic modality has several advantages and has the potential to be expanded to other hard-to-treat bacterial infections such as tuberculosis. First, many potent antibacterials have not been developed or have failed in clinical practice owing to poor pharmacokinetic properties and/or undesired host toxicity. The AAC platform has the potential to revive antibacterials that otherwise do not have suitable profiles as unconjugated drugs. As shown for the *S. aureus* AAC, conjugation to an IgG1 can significantly improve the pharmacokinetics of an antibacterial [3]. For molecules where systemic administration of the unconjugated antibacterial agent results in unacceptable levels of toxicity to normal cells, AACs have the potential to enhance the therapeutic index by maximizing efficacy and minimizing off-target toxicity. In this case the ACC enables targeted delivery of the antibacterial to the surface of the intended bacteria. Internalization of the bacteria–AAC complex then leads to high intracellular free antibacterial concentrations to kill the bacteria, but largely spares non-target host tissue.

**Key Figure**

**Characteristics of Antibody–Antibiotic Conjugates (AACs)**

**Antibody (and the antigen it recognizes)**
- Antigen is well-characterized; consider essential bacterial viability or virulence preferably
- Antigen is consistently abundant and expressed on the surface of bacteria in all phases of infection; limited tendency for antigen-negative variants to emerge
- Lack of high levels of soluble antigen
- Antibody is specific to antigen only on target cells (minimal non-specific binding)
- Antibody maintains characteristics, such as binding specificity and good pharmacokinetics when linked to requisite number of antibiotics

**Antibiotic**
- Highly potent since delivery is limited by antigen copy number
- Non-immunogenic
- Long residence time once released within the host cell
- Bactericidal against cells in dormant state

**Linker**
- Bioorthogonal property: highly stable in circulation but quickly and efficiently degrades to release the active antibiotic once internalized
- Does not adversely impact properties of the antibody or antibiotic

Figure 5. The cartoon describes the optimal AAC characteristics of (i) the antibody (and antigen), (ii) the antibiotic, and (iii) the linker.
from exposure to the toxic antibacterial [3]. This property should permit long-term therapy with an AAC to target persistent infection with minimal antibiotic side-effects.

Second, the use of broad-spectrum antibiotics to treat infections could result in collateral damage because these antibiotics indiscriminately kill both pathogens and commensals. The latter results in dysbiosis of the gut microbiota that has been associated, for example, with outbreak of C. difficile infection, and with various diseases such as inflammatory bowel disease, obesity, and atherosclerosis [51–53]. This has thus led to the development of strategies to generate pathogen-specific antibiotics. When a broad-spectrum antibiotic is linked to the AAC, however, its side effects might be potentially minimized. As an AAC, the antibody that delivers antibiotic to the target organism determines its specificity, and active antibiotics are released only in close proximity to the target organism within the host cells. Thus, when the broad-spectrum antibiotic enters the circulation, it may have undergone orders-of-magnitude dilution, thus not resulting in much damage to the gut microbiota. Consequently, the AAC has the potential of transforming a broad-spectrum antibiotic into a pathogen-specific antibiotic because of the specificity endowed by the antibody.

Third, in principle, the release of free active antibiotic from the AAC does not need to be restricted to intracellular compartments. A linker that is cleaved by an enzyme specifically secreted by the pathogen, but that is otherwise stable in circulation, could potentially be part of an AAC design. A potential application of such a linker might be for bacterial biofilms, where an antibody that recognizes specific antigens on the biofilm might concentrate AACs to these sites, and the biofilm- or bacteria-specific enzyme could cleave the linker to release a high local concentration of active antibiotic at the biofilm.

Several challenges to the development of AACs remain, some of which may be unique to S. aureus AACs (see Outstanding Questions and Box 3). For example, unlike ADC payloads,

**Box 3. Clinician’s Corner**

Common infections are increasingly caused by organisms that are resistant to antibiotics, in part because of poor antibiotic stewardship and minimal investments in antibiotic discovery and development.

Many antibiotics currently in use also adversely affect beneficial members of the gut microbiota. Disruption of healthy microbiota not only enables colonization by antibiotic-resistant pathogens such as Clostridium difficile, vancomycin-resistant enterococci (VRE), MRSA, and multidrug-resistant Gram-negative Enterobacteriaceae, but is also associated with various diseases such as inflammatory bowel disease, obesity, and atherosclerosis [51–53].

On the one hand, targeted antimicrobial therapies can overcome some of these challenges; many potent antibiotics have broad-spectrum antimicrobial activity. On the other hand, many monoclonal antibody therapies have not been successful in the clinic, though they afford exquisite specificity for the microbial target.

However, a recent study has shown that appending a potent antibiotic to a monoclonal antibody against Staphylococcus aureus in the form of an antibody–antibiotic conjugate (AAC) can effectively eradicate the overall infectious burden in several preclinical settings.

AACs, by the nature of their design, have proved to be very effective in killing intracellular bacteria that are otherwise recalcitrant to conventional antibiotic treatments [2]. Extracellular bacteria tagged with these AACs carry with them large amounts of a prodrug (an antibiotic) when they enter into cells. Within the confines of phagolysosomes, host proteases cleave the linked payload to release active antibiotics in high local concentrations to kill the pathogen. This can effectively put an end to the vicious cycle of the intracellular–extracellular hide-and-seek mechanism utilized by several pathogens.

Establishment of intracellular infections has been attributed to long-term recrudescence and failure of conventional antibiotics. Therefore, ablating the intracellular pathogen pool may be key to clinical success. A novel antimicrobial platform such as AAC may be beneficial not only to treat diseases such as S. aureus bacteremia but also other deadly and hard-to-treat infections such as tuberculosis.
which are highly potent, typically with inhibitory concentration 50% (IC50) values in the picomolar to submicromolar range, antibiotics are generally much less potent [34]. The amount of antibiotics that could be delivered to bacteria is dictated by the abundance of the antigen on bacterial cell surfaces. For the S. aureus AAC with a antibiotic:antibody ratio of 2, the amount of antibiotic delivered is twofold higher than the number of epitopes. Future efforts should explore technologies that permit the attachment of more antibiotics per antibody without introducing aggregation problems and negatively impacting on the pharmacokinetic properties of the AAC. Therefore, to design an efficacious AAC, selection of antibiotic, antibody, and linker, as well as conjugation strategies to maximize binding, internalization, and payload release, are of paramount importance. Finally, AACs are complex molecules and can be challenging to manufacture. However, technological and manufacturing advances attained from the production and clinical use of ADCs could potentially be brought to bear for future product development of AACs.

Concluding Remarks

In the face of rising antibiotic resistance and an innovation gap in the discovery of novel antibiotics, the AAC platform, by combining pharmacological attributes of both antibody and antibiotics into a single molecule, offers an exciting opportunity for a novel therapeutic modality to treat infectious diseases. The AAC has the potential to deliver potent antibacterial compounds that may not have a suitable profile as unconjugated drugs, such as poor pharmacokinetics or low therapeutic index. Finally, where poor antibiotic efficacy and disease relapse have been associated with the ability of a variety of bacterial pathogens to survive within host cells [54], the AAC platform promises to enhance antibiotic efficacy against these infectious diseases.

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Supplemental information

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Outstanding Questions

Can AACs be used against pathogens if they have an exclusive extracellular lifestyle or if the pathophysiology of S. aureus and its intracellular lifestyle is not clinically relevant? Current AACs use an intracellular protease to release antibiotics in the phagolysosomal environment. In principle, extracellular pathogens can be eradicated by rendering them with AACs into phagocytic environments through antibody-mediated intracellular uptake for eventual killing. Alternatively, AACs might be used to localize the potent antibiotic payload to the site of infection, such as biofilm, where the release of antibiotics following cleavage of the linker by a pathogen-secreted protease, crucial for virulence, could lead to eradication of pathogens at these sites.

Could the AAC be used as a therapeutic platform to revive potent antibiotics that failed owing to unwanted toxicity in humans or exhibited poor pharmacokinetic properties as a free drug? Because AACs release free antibiotics and concentrate them within cells or at the site of infection, the overall amount of antibiotics to be delivered as an AAC would be lower than if the antibiotic were to be administered as a stand-alone therapy. Thus, could antibiotics that have failed in the clinic owing to increased toxicity be used in lower amounts to minimize such side effects? Similarly, can we rescue antibiotics that have poor pharmacokinetics using the AAC approach, especially if the pharmacokinetics are significantly improved by the antibody? This could be a potential avenue to bring novel antibiotics into the market.

What is the additional mechanism of action of dmDNA31, the antibiotic currently used in the above-mentioned proof-of-concept studies? dmDNA31 is closely related to the rifampicin and related ansomycin class of antibiotics. However, unlike rifampicin, dmDNA31 can kill dormant and persistor bacteria. Therefore, in addition to its mode of action through the β subunit of bacterial RNA polymerase (RpoB), dmDNA31 must impart other microbiocidal properties. Does is perturb bacterial cell membranes, or alter the electron potential of bacterial membranes? Further clues into this additional mechanism of action could help to reveal novel antibiotic classes to fight intracellular or persister bacteria.
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Could the S. aureus AAC be used as a standalone treatment for invasive infections? Although dmdN3A1 possesses an additional mode of action that enables it to kill dormant and persister bacteria, does its engagement of bacterial RpoB bring with it resistance liabilities commonly seen among the rifampicin class of antibiotics targeting RpoB? Could this potential liability be overcome by combining this strategy with other standard-of-care antibiotics?

Does the engagement of Fc receptors by the AAC-bacteria complex influence the clinical outcomes of infection? The antibody of the S. aureus AAC retains Fc function and is capable of engaging both activating and inhibiting Fc receptors that would have downstream impact on inflammation. How much does this Fc-FcR interaction contribute to the ultimate outcome of treatment with AAC?


