Clinical Pharmacology and Translational Considerations in the Development of CRISPR-Based Therapies

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Genome editing holds the potential for curative treatments of human disease, however, clinical realization has proven to be a challenging journey with incremental progress made up until recently. Over the last decade, advances in clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) systems have provided the necessary breakthrough for genome editing in the clinic. The progress of investigational CRISPR therapies from bench to bedside reflects the culmination of multiple advances occurring in parallel, several of which intersect with clinical pharmacology and translation. Directing the CRISPR therapy to the intended site of action has necessitated novel delivery platforms, and this has resulted in special considerations for the complete characterization of distribution, metabolism, and excretion, as well as immunogenicity. Once at the site of action, CRISPR therapies aim to make permanent alterations to the genome and achieve therapeutically relevant effects with a single dose. This fundamental aspect of the mechanism of action for CRISPR therapies results in new considerations for clinical translation and dose selection. Early advances in model-informed development of CRISPR therapies have incorporated key facets of the mechanism of action and have captured hallmark features of clinical pharmacokinetics and pharmacodynamics from phase I investigations. Given the recent emergence of CRISPR therapies in clinical development, the landscape continues to evolve rapidly with ample opportunity for continued innovation. Here, we provide a snapshot of selected topics in clinical pharmacology and translation that has supported the advance of systemically administered *in vivo* and *ex vivo* CRISPR-based investigational therapies in the clinic.

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) was originally identified as an adaptive immune system in bacteria and has since been developed for editing eukaryotic cellular DNA.[1] Just over 1 decade ago, a landmark paper reported how CRISPR-associated nucleases could be programmed to execute a precise cut at specific DNA sites.[1] In the relatively short time that has followed, multiple successive advances have unlocked the genome editing potential of CRISPR for human therapeutic use. From **Figure 1**, genome editing now represents the latest in a series of advances in treating human disease.

CRISPR therapies may be subcategorized into both *in vivo* and *ex vivo* applications.[2,3] For *in vivo* applications, CRISPR therapies are administered locally or systemically to the patient. In the *ex vivo* setting, a particular cell type is typically first isolated and expanded prior to exposure to the CRISPR therapy; the edited cells are then introduced to the patient. Although *ex vivo* CRISPR therapies have had an initial head start in development, *in vivo* CRISPR therapies are becoming an increasingly important component of the genome editing clinical landscape. A recent 2023 review,[3] provides a comprehensive tabulated summary of CRISPR clinical trials and summarizes 28 clinical trials investigating *ex vivo* therapies and 6 clinical trials investigating *in vivo* CRISPR therapies.

Various isoforms of CRISPR/Cas nucleases have been isolated for use in medical applications.[4] From **Figure 2**, one set of therapies enlists Cas9 (derived from *Streptococcus pyogenes*), which can be directed by short RNA sequences that act as guides (sgRNA) to recognize a complementary target DNA sequence next to a protospacer adjacent motif sequence. The nuclease then introduces a double-stranded break (DSB) in the DNA. For permanent gene knockout, following cleavage, endogenous DNA repair mechanisms rejoin the ends of the cut and introduce insertions or deletions (collectively referred to as "indels") that result in decreased production of protein. Similarly, for targeted gene insertion aimed at gain-of-function, when provided with a recombinant adeno-associated virus (AAV) template sequence, such as a protein-coding sequence, the cell's repair processes can incorporate that novel function sequence into the genomic DNA at the site of the break. For insertion of the native or wild-type gene, the CRISPR guide facilitates insertion of the functional gene at a specific location in the genome that could have minimal impact to the function of the recipient gene locus.

Several important features serve to differentiate CRISPR therapies from previous classes of drugs, especially as they pertain to translation and clinical pharmacology. First, as a nucleic acid-based therapy, targets for CRISPR therapies occupy an orthogonal space relative to

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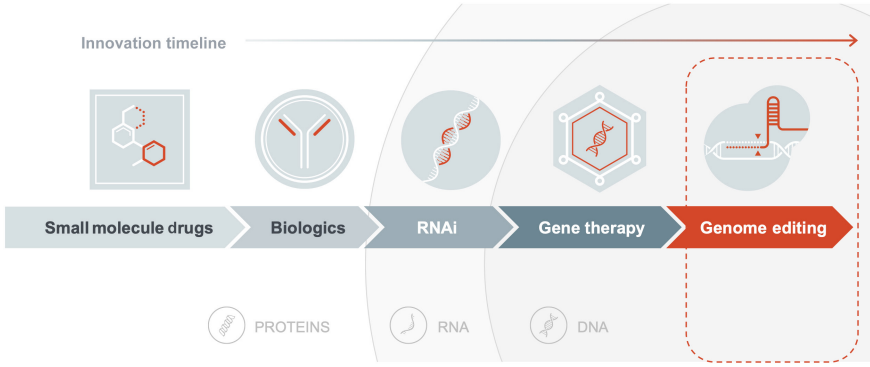


Figure 1 Genome editing represents the latest in a series of advances in treating human disease.

those for small molecules and biologics, which are more commonly restricted to the binding domains of proteins and extracellular proteins, respectively. Second, successful *in vivo* clinical application of CRISPR therapies relies critically upon delivery vehicle, resulting in special bioanalytical and immunogenicity considerations. Further, the absorption, distribution, metabolism, and excretion (ADME) characteristics of the delivery vehicle can act to govern those of the CRISPR-based therapeutic, especially prior to uptake by the targeted cell population. Third, CRISPR therapies aim to make permanent gene knockout or gene insertion and achieve therapeutically relevant effects, potentially with a single dose. This contrasts with prior classes of therapies where chronic administration is required to maintain a steady state pharmacodynamic (PD) effect.

Here, we review selected facets of the development of systemically administered CRISPR therapies, especially as they pertain to clinical pharmacology and translation. The review is first organized by *in vivo* and *ex vivo* application and is further subdivided by selected topics as they pertain to these applications.

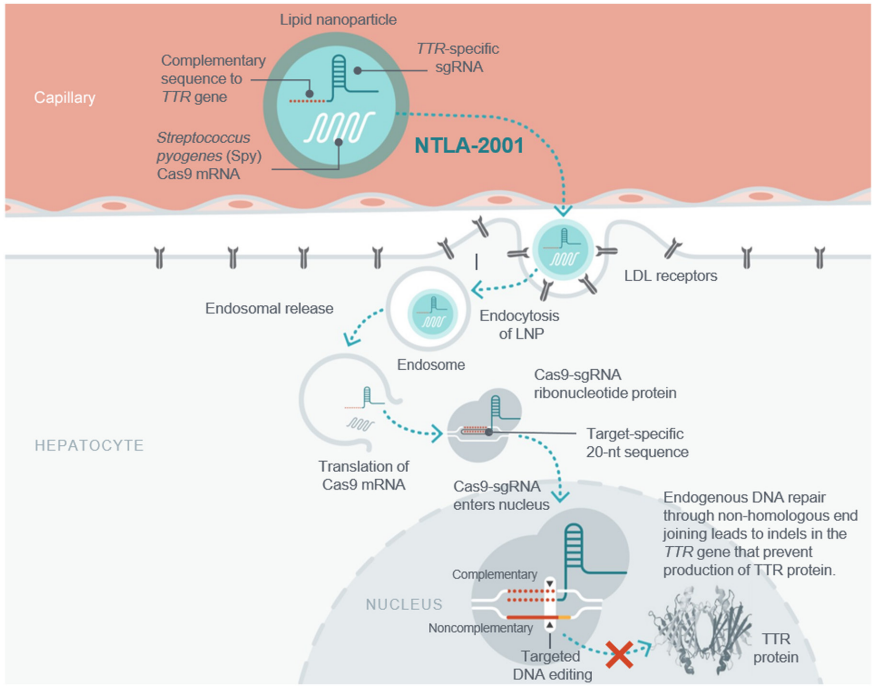


Figure 2 The mechanism of action for NTLA-2001. NTLA-2001 is comprised of a lipid nanoparticle (LNP) which in turn encapsulates both messenger RNA (mRNA) molecule encoding *Streptococcus pyogenes* clustered regularly interspaced short palindromic repeats-associated protein 9 (Cas9) protein and a single guide RNA (sgRNA) molecule specific to the human gene encoding transthyretin (TTR). Proceeding from the top down, following systemic administration, NTLA-2001 is taken up by the liver. Entry into hepatocytes is expected to be facilitated by interaction with low density lipoprotein (LDL) receptors followed by endocytosis. The Cas9 mRNA and sgRNA are released into the cytoplasm where Cas9 mRNA is translated to Cas9 enzyme. The sgRNA associates with Cas9 protein to yield a Cas9-sgRNA ribonucleoprotein complex (RNP). The RNP then enters the nucleus where it recognizes the protospacer-adjacent motif (PAM) on the noncomplementary DNA strand. A target-specific sequence of the sgRNA binds to the target site, leading to confirmational changes in the Cas9 protein and ultimately cleavage of both strands of the DNA. Endogenous mechanisms repair the ends of the cut, potentially introducing insertions or deletions (collectively referred to as "indels") that result in decreased production of TTR protein.

IN VIVO CRISPR THERAPIES

Novel formulations and bioanalytical characterization

Several barriers preclude direct administration of therapeutic nucleic acids *in vivo*, including those which act to rapidly clear nucleic acids from the systemic, stimulate immune response, and limit cellular uptake.[5] Early attempts to deliver CRISPR/Cas9 leveraged historical approaches from gene therapy, including viral vector delivery systems.[6] Finn *et al*.[7] subsequently identified four characteristics for next-generation platforms that were better suited to therapeutic application of CRISPR/Cas9. These characteristics included platforms with transient Cas9 expression, efficient delivery of both Cas9 messenger RNA (mRNA) and sgRNA, flexibility for multiple dose administration, and scalability for eventual commercial production. A formulation based upon the lipid nanoparticle (LNP) was proposed to fulfill these criteria, and an LNP-based formulation was ultimately used for NTLA-2001, an investigational therapy and the first systemically administered *in vivo* CRISPR/Cas9-based therapy evaluated in the clinic.[8] Although systemically administered *in vivo* CRISPR-based therapies rely upon both AAV- and LNP-based delivery vehicles, the LNP predominates the clinical investigation landscape at the time of writing[3]; the primary focus in the sections that follow is accordingly upon systemic administration of the LNP-based system.

With the novel formulations currently under clinical investigation comes a host of considerations for robust bioanalytical characterization. As summarized elsewhere,[9] bioanalytical considerations for AAV vectors include those related to both biodistribution and shedding, with biodistribution pertaining to the disposition of AAV and shedding defined explicitly as how AAV is excreted or released from the patient's body.[10,11] Patisiran, a small interfering RNA (siRNA)-based therapy indicated for hereditary transthyretin (ATTR) amyloidosis, provides an illustration of the bioanalytical characterization of an LNP-based formulation for a nucleic acid therapy. Zhang *et al*.[12] report the pharmacokinetics (PKs) of three analytes following administration of patisiran to patients with hereditary ATTR amyloidosis; two of the analytes corresponded to lipid excipients of the LNP (DLin-MC3-DMA and PEG2000-C-DMG), whereas the remaining analyte corresponded to drug substance (i.e., the siRNA component). Accordingly, a complete bioanalytical characterization of a novel nanoparticle formulation can entail multiple analytes and is a consideration not only for characterization of the PKs and distribution of CRISPR/Cas9-based therapy, but also PK/PD relationships.[13] The corresponding set of bioanalytical methods to support this characterization is based on context of use (COU) using fit-for-purpose technical platforms, such as quantitative polymerase chain reaction (PCR)/reverse transcription PCR, droplet digital PCR, ligand binding assays (such as enzyme-linked immunosorbent assay) and mass spectrometry.[11] The COU-driven method validation or qualification is used to demonstrate that methods are suitable for intended use.[14]

Absorption, distribution, metabolism, and excretion

The determinants of ADME for systemically administered LNP-based CRISPR therapies are largely determined by those of the corresponding nanoparticle formulation administered as an intravenous (i.v.) therapy. **Figure 3** summarizes several important aspects of nanoparticle ADME. Following i.v. administration, nanoparticles are exposed to a myriad of cells and biomolecules present in the vascular system as they distribute to additional tissues and organs of the body. Varying degrees of protein adsorption can occur on the nanoparticle surface as it transits the vascular compartment.[15] Depending on the nature and extent of protein adsorption, the resultant protein corona can become an important contributor to the distribution, cellular uptake, elimination, and, ultimately, activity of the nanoparticle formulation. Nanoparticles may be cleared from the circulation by the mononuclear phagocyte system (MPS), or the renal system or hepatobiliary systems.[13] Opsonins comprise a subset of the proteins that can adsorb to the surface of nanoparticles, and opsonization acts to tag these nanoparticles for sequestration and processing by the phagocytic cells of the MPS. Nanoparticle elimination via the renal route is especially dependent on the hydrodynamic diameter of the nanoparticle; the hydrodynamic diameter is in turn a function not only of the *in vitro* particle diameter, but also the protein corona that can form upon *in vivo* administration. A recent investigation in mice, who were administered a series of nanocrystals of increasing size, suggested particles of hydrodynamic diameter of 5.5 and lower were renally excreted, whereas the 8.65 nm particles were not.[16] Although too large of a hydrodynamic diameter may preclude renal excretion of the intact nanoparticle, this route remains available for individual components of the nanoparticle that can result from degradation. Larger particles that have not already been cleared by phagocytosis are subject to elimination by the liver to the feces.

Nanoparticles that remain in circulation are then available for a succession of transport, cellular uptake, and trafficking events that culminate with release of the nucleic acid cargo in the cytosol. As is the case with all systemically administered drugs, the ability of the nanoparticles to access the target organ is governed first by tissue perfusion. More unique to the case of nanoparticles is the set of mechanisms available for transvascular transport and extravasation. Nanoparticles are believed to extravasate, either by transport through intracellular fenestrations or via transcytosis. Much of the quantitative understanding of nanoparticle extravasation comes from studies in tumors. A mathematical model for the flux of material crossing a vessel wall includes a term for vascular permeability, which is in turn a function of the characteristics of the particle and the vessel wall; vascular permeability falls to zero when the nanoparticle size is in excess of the pore cutoff size of the vessel, illustrating the significance of particle characteristics on extravasation.[17] Once extravasated, interstitial transport is governed by a combination of convective and diffusive mechanisms that, depending on the nature of the extracellular matrix, can act to restrict tissue penetration to sites far from the vessel wall. Once extravasated and upon successful navigation of the interstitium, the nanoparticle is available for cellular uptake most commonly by endocytosis. Endosomal escape of the nucleic acid cargo becomes the next essential step[18] prior to the downstream events in the cell summarized in subsequent sections of this review.

As a well-perfused organ with fenestrated endothelium, the liver is especially well-suited as a targeted organ for systemic nanoparticle delivery. This, combined with the liver's role both in the generation and elimination of serum proteins, has driven interest in the liver as

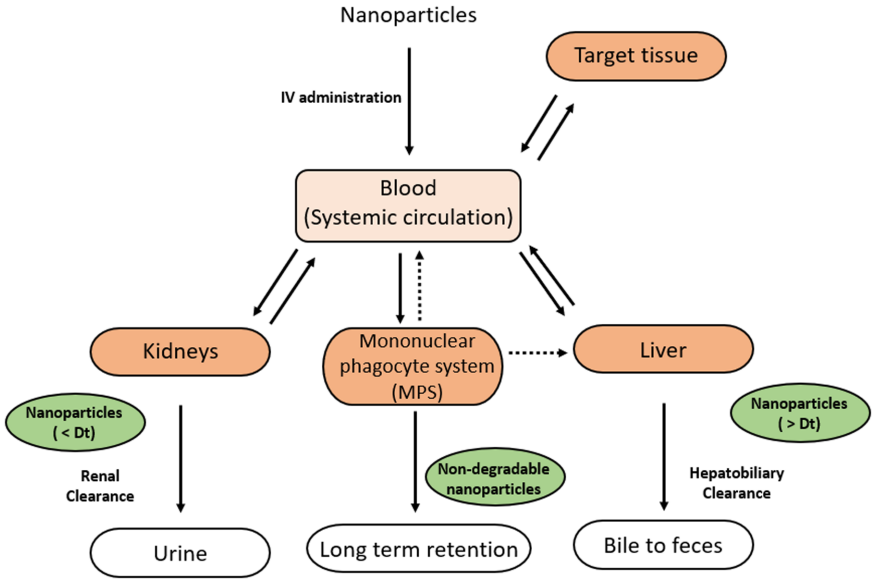


Figure 3 Schematic representation of absorption, distribution, metabolism, and excretion processes for nanoparticles following intravenous (i.v.) administration. Following i.v. administration to the systemic, nanoparticles both distribute to the targeted tissue and are cleared from the circulation by the mononuclear phagocyte system (MPS), or the renal or hepatobiliary routes. Factors such as nanoparticle size and opsonization are important determinants of the importance of these clearance routes. Opsonization of nanoparticles is an important first step in the process of sequestration and processing by the MPS. The hydrodynamic diameter of the nanoparticle is influenced both by the particle diameter and the protein corona. Particles below a threshold hydrodynamic diameter (Dt) are available for renal excretion, as are individual degradation products of the nanoparticle. Larger particles are then available for elimination by the liver to the feces. Adapted from ref. 92.

a targeted organ for gene therapy. Especially given the customizability, scalability, and potency of LNP-based formulations, the LNP is viewed as the current state-of-the-art for liver targeting.[7] Patisiran provides an illustrative example of the clinical ADME characteristics of an LNP-based liver-targeted gene therapy. The patisiran LNP is comprised of four components, including ionizable amino lipid DLin-MC3-DMA and a polyethylene glycol (PEG) lipid 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (in addition to 1,2-distearoyl-sn-glycero-3-phosphocholine and cholesterol).[13] Liver targeting is important for patisiran in ATTR amyloidosis given the role of misfolded transthyretin protein (TTR) in the disease pathobiology and that TTR is almost exclusively manufactured in the liver.[19] Liver uptake of the LNP is governed by a series of events.[12,20,21] First, the PEG-lipid component disassociates from the LNP, which drives opsonization of the LNP by apolipoprotein E. The LNP then enters the liver through vascular fenestrations, where it becomes available for low-density lipoprotein receptor-mediated endocytosis by hepatocytes.[19,22,23] There are at least two fates for the internalized LNP. One fraction of the LNP releases its cargo into the cytoplasm, leading to knockdown and downstream reductions in TTR protein. Another fraction of the LNP is believed to undergo exocytosis, leading to re-entry into the circulation. The resultant plasma disposition for the RNA component and DLin-MC3-DMA was multiphasic and was characterized with an initial rapid decline from peak plasma concentrations, followed by a secondary peak and an elimination phase. Negligible amounts of the RNA component were detected in urine, whereas low levels of a metabolite of DLin-MC3-DMA (4-dimethylaminobutyric acid) were reported in urine.

The encyclopedic reviews of Chen[11] and Sun[24] summarize the determinants of AAV biodistribution and shedding. One noteworthy consideration for AAV comes from the capsid serotype, which plays an important role in both tissue tropism and in shedding. Another consideration unique to AAV comes from vector shedding and the potential risk for transmission from treated to nontreated individuals. Accordingly, clinical shedding assessments are commonly conducted during clinical investigation, involving collection and assay of excreta and secreta (e.g., urine, feces, saliva, nasal fluid) and blood and semen.[10] However, these theoretical concerns are tempered somewhat in the context of replication-incompetent recombinant AAV because no new particles can be produced. Accordingly, available data suggest that following i.v. administration of AAV-5, -8, and -9 to humans, vector DNA levels were either near the assay limit or at undetectable levels within the first 6 months postdose in multiple secreta and excreta matrices.

On-target/off-target editing

A DSB signals the final outcome of CRISPR/Cas9 activity and the beginning of endogenous DNA repair. Repair structures are most commonly formed through either non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ has a tendency to gain or lose small amounts of genetic material that can disrupt genomic regulation, structural features, or coding elements.[25] HDR is a higher-fidelity repair process that requires a template to supply the correct sequence of the original DNA strand.[26] Programmable nucleases are used to introduce appropriately positioned DSBs and trigger a sequence of DNA repair events, resulting in a permanent, location-specific change. The expected outcome of therapeutic genome editing is a permanent alteration in genomic DNA sequence.

The genome editing field is actively assessing factors governing editing accuracy. Inaccurate or "off-target" editing can potentially

lead to genotoxicity and considered potentially linked to a variety of unforeseen consequences. The primary driver of off-target mutations resulting from programmable nucleases is the presence of DNA sequences elsewhere in the genome that are highly similar to the intended target locus. It is crucial to evaluate the likelihood of unintended biological effects by characterizing potential off-target sites. Targeting unique genomic sequences acts to reduce off-target mutations caused by programmable nucleases. Whole-genome sequencing can detect off-target mutations but is impractical for nonclinical or clinical studies due to sample input requirements. Instead, a stepwise approach can be used to identify and focus on the most likely off-target locations, allowing for increased sequencing depth and sensitive detection of rare mutations. This approach increases confidence in confirming or ruling out off-target activity at candidate sites.

Off-target discovery aims to identify genomic locations where editing may occur outside the intended site using multiple methods. Computational prediction based on sequence homology and biophysical binding rules is widely used, but not all off-target sites can be reliably identified *in silico*.[27,28] Empirical approaches, such as cell-free and cell-based methods, can be used to complement *in silico* prediction.[29,30] The combined set of *in silico* and empirically identified off-target sites can be categorized into intergenic, intronic, and exonic regions. Risk assessment begins by considering the functional genomics of potential off-target loci. Exonic regions carry the greatest risk due to their role in protein-coding sequences. Intronic regions are considered lower risk, but attention should be paid to possible splice sites and regulatory features. Intergenic regions are considered the lowest risk. The review of potential off-target sites identifies any genes with known roles in cellular proliferation or oncogenic effects. If validated off-target activity occurs within a gene, the impact of losing expression can be projected, biodistribution data can inform risk, and de-risking experiments may be warranted to assess the influence of editing on off-target gene expression.

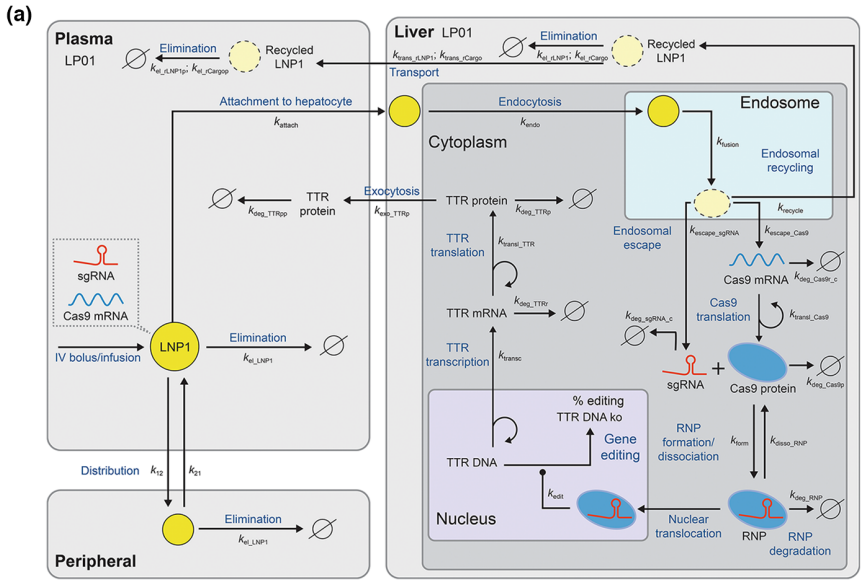
Off-target verification subjects the list of potential off-target sites, generated during discovery, to deep sequencing. The objective is to sensitively confirm the absence or presence of indels potentially formed by off-target genome editing activity. Focusing sequencing depth to a list of the most likely off-target locations increases sensitivity for detecting low editing rates and improves coverage. Genetic sample material should be representative of the therapeutic approach with multiple tissue/cell donors tested to improve confidence in identifying rare off-target events. Biodistribution data may provide additional information for systemically administered products. Animal models have limited relevance due to incomplete genomic homology, and quality metrics can be used to compare interpretation for each candidate site in an appropriate context relative to all other sites genome wide.

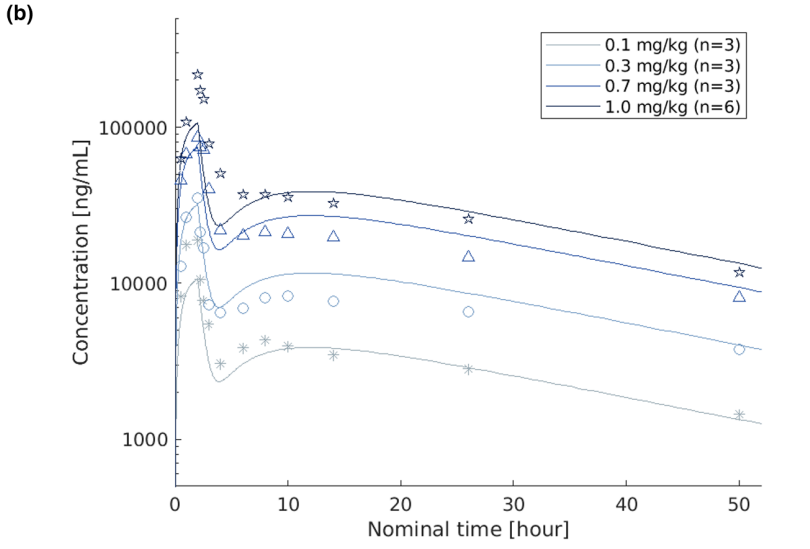
An alternative unintended DNA repair outcome is structural variation (SV). The SVs are most common at the on-target site of simultaneous DSBs on sister chromatids.[31] Sister chromatid exchange without indel formation represents a balanced rearrangement and not expected to have functional consequence. The potential for chromosomal SV increases with editing activity and with the number of sites simultaneously edited. The intentional generation of a deletion through complex editing at multiple on-target sites may also result in unintended effects, such as inversions, acentric and dicentric chromosomes, and inter-chromosomal translocations. The possibility of unwanted chromosomal SVs should be considered when using programmable nucleases for gene or cell therapy.

Pharmacodynamics

An emerging literature database suggests both subtle and more apparent differences regarding the nature of PD responses following CRISPR administration relative to the previous classes of therapies of **Figure 1**. One seemingly subtle difference is reflected in the nature of the dose-PD relationship. On a per-cell basis, the PD response would not necessarily be expected to follow a continuum but would instead theoretically be expected to be one of a set of discrete values. For example, for a given diploid cell, one would theoretically expect 0, 50%, or 100% knockout of the alleles.[32] However, PD data obtained from a population of cells would reflect averaging across the individual discrete states of the cell population and not be expected to exhibit this degree of discretization. Instead, accumulating data suggest that the dose-PD relationship both *in vitro* and *in vivo* resembles the familiar Michaelis-like, saturating dose-PD relationship. An early clinical example of the nature of dose-PD response comes from NTLA-2001, which aims to treat ATTR amyloidosis by reducing the circulating levels of TTR. The saturating nature of the dose–response relationship for NTLA-2001 above dose levels of 0.3 mg/kg was evident following a single administration of NTLA-2001 0.1 to 1 mg/kg, with TTR levels that were reduced by ≥ 90% of baseline by day 28 following the 2 top doses of 0.7 and 1.0 mg/kg NTLA-2001 (**Figure 4c**).[33] A more striking feature of the PD response for CRISPR comes from the duration of the response. Although chronically administered drugs are characterized by fluctuations in both circulating levels of drug and the downstream PD, CRISPR therapies aim to make permanent gene knockout or gene insertion resulting in deep, consistent, and durable responses after a single administration and well after PK washout. Returning to the findings in patients with ATTR amyloidosis with cardiomyopathy (ATTR-CM), TTR levels were reduced by ≥ 90% of baseline by day 28, following NTLA-2001 0.7–1.0 mg/kg, and these reduced levels continued to be maintained at 4–6 months of follow-up (i.e., the date of the data cutoff).[34] A similar PD response was likewise observed for NTLA-2002, an investigational CRISPR/Cas9-based therapy targeting *kallikrein B1* (*KLKB1*) in development for hereditary angioedema (HAE); following administration of 25 and 75 mg NTLA-2002 to patients with HAE, rapid plasma kallikrein reductions of 65% and 92% at nadir, respectively, persisted for the duration of reported follow-up.[35] Most notably, in both instances, this prolonged PD duration greatly dwarfed the apparent terminal PK half-life of 20–25 hours for the ionizable lipid component of the LNP, LP01.[33]

In addition to durable PD that characterizes gene knockout, preclinical data suggest durable expression following targeted gene insertion. Following administration of a conventional recombinant AAV delivery system, double-stranded AAV genomes circularize via their inverted terminal repeats and can become episomes which persist extra





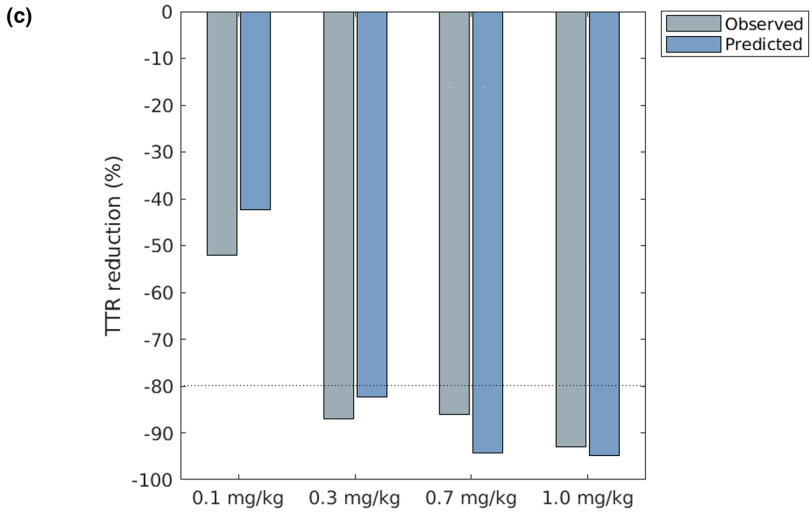


Figure 4 The quantitative systems pharmacology (QSP) model for NTLA-2001. (**a**) The QSP model is comprised plasma, liver, and peripheral compartments. The model describes the plasma disposition and subsequent endocytosis of the NTLA-2001 by hepatocytes. Once endocytosed, the lipid nanoparticle (LNP) fuses to the endosomal membrane at which point a fraction the LNP gets recycled out of the cell and a portion of the LNP cargo (guide RNA (sgRNA) and clustered regularly interspaced short palindromic repeats-associated protein 9 (Cas9) messenger RNA (mRNA)) further escapes into the cytosol. In the cytosol, the Cas9 mRNA is translated into protein and reversibly combines with the sgRNA to form a ribonucleoprotein (RNP). Combined into one reaction in the model, the RNP translocates into the nucleus, binds to the transthyretin (TTR) DNA, and makes a cut forming the knockout species of the TTR DNA (TTR DNA ko). TTR DNA that remains uncut is transcribed into TTR mRNA which then gets translated into the TTR protein. See **Table S1** for definition of rate constants. (**b**) Mean of observed (points, see legend)[32] vs. QSP-model predicted (lines) LP01 PK data following administration of 0.1–1.0 mg/kg NTLA-2001 to TTR patients in first-in-human (FIH) study ITL-2001-CL-001. (**c**) Mean of observed[32] vs. QSP-model predicted TTR reduction following administration of 0.1–1.0 mg/kg NTLA-2001 to TTR patients in FIH study ITL-2001-CL-001. The dashed line corresponds to 80% TTR reduction.

chromosomally in the nucleus.[11] Although the episomal DNA can provide long-term, promoter-driven gene expression in non-dividing cells, the DNA becomes diluted in dividing cells. NTLA-3001 is an investigational transgene insertion candidate intended for the treatment of alpha-1 antitrypsin deficiency-associated lung disease, and is composed of both LNP and promoterless AAV components for transient Cas9 delivery and insertion template delivery, respectively.[36] In this way, NTLA-3001 harnesses the CRISPR-mediated DSB to drive insertion of the transgene that can then leverage an endogenous genomic promoter for transcription and protein expression, preventing transgene dilution in dividing cells and obviating the need for an exogenous promoter to stably express the transgene. Accordingly, investigations in nonhuman primate models suggest durable physiologic levels of human A1AT can be achieved after a single dose.

Immunogenicity

Immunogenicity considerations for CRISPR therapies are driven predominantly by the nature of the delivery vehicle (AAV and LNP) and transgene product including Cas9 protein. Both innate and adaptive immune responses can affect the safety and efficacy of AAV vector-mediated gene transfer in humans, in some cases, resulting in acute toxicities, such as infusion-related reactions, hepatotoxicity, and thrombotic microangiopathy (TMA).[37–39] The AAV capsid-associated immunogenicity is believed to be dose-dependent.[40] Strategies to minimize immunogenicity include improvement of AAV capsid critical product attributes, such as purity (i.e., reducing empty capsid) and limiting the total AAV dose to below 10^{14} vg/kg. Codon optimization to reduce or eliminate cytosine-phosphate-guanine motifs in the transgene has demonstrated reduction of the T cell response via toll-like receptor, such as TLR9.[41] For patients with a higher level of pre-existing anti-AAV antibodies, hyper-immune status, or both, the use of rituximab and calcineurin inhibitors in addition to steroids can possibly reduce immunogenicity. Similarly, TMA can be managed with the anti-C5 antibody eculizumab. The use of empty capsid as a decoy to mitigate pre-existing humoral immunity is not recommended.[42–44]

The LNPs have been used as a delivery vehicle for a variety of mRNA-based therapies. In principle, a single i.v. dose is considered to have a lower immunogenicity risk than multiple doses via subcutaneous or intramuscular routes. Based on knowledge from pegylated biologics, the PEG component of the LNP is a known immunogenic epitope. Given the environmental exposure to PEG (from food and cosmetics) and coronavirus disease (COVID) mRNA LNP vaccines, an assessment of the anti-PEG antibody response is warranted. There does not appear to be a definitive association of anti-PEG antibody post-LNP dose to severe adverse events. As an example, patisiran has shown low incidence of anti-PEG antibody response with no reported impact on PK, PD, or safety.[45]

Regarding the transgene product, if the mRNA codes for protein with a high level of expression, especially in patients who do not have endogenous protein due to genetic mutations, monitoring anti-transgene product antibody becomes critical. If the transgene contains Cas9 mRNA of bacterial origin, the Cas9 protein could elicit both T and B cell immune responses, in addition to any pre-existing immunogenicity due to prior exposure to *Streptococcus* or *Staphylococcus*. Monitoring of anti-Cas9 protein antibody response is recommended for patients receiving Cas9 mRNA, along with a complementary evaluation of the potential impact of antibody on PK, PD, and safety. The anti-Cas9 protein antibody response is characterized minimally in terms of pre-existing antibodies, which may be present presumably due to prior exposure to bacterial-derived Cas9 proteins, as well as postdose seroconversion rate (incidence) and magnitude of the response (titer). The use of T cell functional tests in patients (such as interferon gamma enzyme-linked immunosorbent spot assay and multiparameter flow cytometry) could be used for further exploratory evaluation, although there are potentially problematic considerations with these assays. These tests require larger blood volume and are associated with high complexity for sample processing to retain viable and functional T cells. Monitoring serum inflammatory cytokines is an alternative approach; it is important to bear in mind that systemic cytokine elevations may reflect a local T cell response when interpreting these data.

Translation

A recent review provides a regulatory perspective regarding model-informed drug development (MIDD) for gene therapies.[46] Several of the unique considerations for gene therapy, including those discussed previously and related to formulation and manufacturing, PK/PD, and single-dose administration, provide an opportunity for innovative applications of MIDD above and beyond those established for previous classes of therapies. Translation of nonclinical PK/PD data from nonclinical species to human is a nascent science in gene therapy. Application of traditional allometric methods based upon observations in nonclinical species is complicated by issues such as species specificity and immunogenicity.[47] Despite these complications, a recent report suggests that a metric capturing the efficiency of viral-based vectors follows an allometric relationship across species for using AAV factor IX data for hemophilia B.[48] It remains to be seen whether these empirical approaches provide similarly robust human PK/PD projections for CRISPR therapies. Accordingly, a semimechanistic quantitative

systems pharmacology (QSP) model was used to project human PK/PD for NTLA-2001.[49] From **Figure 4a**, following i.v. administration, the QSP model captures key determinants governing NTLA-2001 PK/PD, including LNP distribution to the liver and endocytosis by hepatocytes, release of RNAs from the LNP, translation of the Cas9 mRNA into protein, and combination with the sgRNA to form the RNP, RNP translation into the nucleus, DNA cleavage, and subsequent reduced levels of circulating TTR. In addition, a model provision is included to allow a fraction the LNP to undergo exocytosis back into the circulation.[12,50] **Figure 4b** compares the PKs of ionizable lipid LP01 following administration of 0.1–1.0 mg/kg NTLA-2001 to patients with hereditary ATTR amyloidosis with polyneuropathy (ATTRv-PN) in first-in-human (FIH) study ITL-2001-CL-001[33] to QSP model predictions. From **Figure 4b**, the QSP model captures hallmark features of LP01 PKs: a rapid decline from peak followed by a secondary peak (driven by the model provision for exocytosis) and a subsequent log-linear phase. **Figure 4c** compares the corresponding observed TTR reductions to QSP model predictions; from **Figure 4c**, the QSP model further captures the saturating dose–response for NTLA-2001 dose and TTR reduction.

Dose selection

A myriad of challenges has been identified regarding dose selection in gene therapy. The comparatively small trial sample sizes that typify rare disease development can lead to a correspondingly small clinical database to inform dose selection for subsequent stages of development.[51] Similarly, there is a perception of a time lag between the accelerating pace of gene therapy clinical development and quantitative methods to support dose selection in this space.[46,51] Although innovators and early adopters are driving new approaches in this space, dissemination and uptake of these approaches by the broader community is the next step in the diffusion of innovation. Because the clinical development of siRNA therapeutics precedes that of CRISPR therapies, the correspondingly larger literature database for siRNA illustrates the uptake of methods for gene therapy dose selection. A 2022 review summarizes the nature and extent of modeling and simulation approaches in siRNA development and provides a glimpse into quantitative methodologies for dose selection in gene therapy.[52]

In contrast to the mature literature database for siRNA dose selection, the corresponding literature documenting dose selection for systemically administered *in vivo* CRISPR therapies is nascent at the time of writing. The FIH study of NTLA-2001 provides a recent case example of a systemically administered *in vivo* CRISPR therapy that illustrates both starting dose selection and how phase I dose-escalation data have been leveraged to inform dose selection for subsequent stages of development. Gillmore *et al.*[8] summarizes the nonclinical data that informed the design of the FIH study for NTLA-2001. Briefly, in addition to the *in silico* and *in vitro* data package that informed the study, preclinical mouse and cynomolgus studies for NTLA-2001 demonstrated that a single dose of NTLA-2001 (or cynomolgus surrogate) resulted in both durable editing and deep reductions in serum TTR protein at well-tolerated dose levels. Toxicokinetic data obtained in the cynomolgus monkey suggested relatively rapid clearance of the analytes associated with the LNP. The no-observed-adverse-effect level was subsequently determined to be 3 mg/kg in the cynomolgus monkey. The maximum recommended starting dose of NTLA-2001 was determined to be 0.1 mg/kg based on total body surface area scaling and assuming a safety factor of 10. The resultant single ascending dose component of the two-part, open-label, multicenter study explored 0.1, 0.3, 0.7, and 1.0 mg/kg NTLA-2001 in patients with ATTR-PN and 0.7 and 1.0 mg/kg in patients with ATTR-CM.[34] As discussed previously (see Pharmacodynamics) from **Figure 4c**, experience in the dose-escalation segment conducted in patients with ATTRv-PN suggested that there was a saturating dose–response relationship for TTR reductions following administration of NTLA-2001 at doses of 0.1–1.0 mg/kg; further, a semimechanistic QSP model appeared to capture this underlying relationship.[49] Findings following administration of NTLA-2001 at 0.7 and 1.0 mg/kg to patients with ATTR-CM were generally consistent with those reported in ATTRv-PN, with mean TTR reductions of > 90% for both doses by day 28 that were sustained through the data cutoff (4–6 months). Further, NTLA-2001 was generally well-tolerated across this dose range, and prior PK simulations suggested that continued adjustment by body size in the expansion was not necessary.[33] Accordingly, the 55 mg dose (fixed dose equivalent of 0.7 mg/kg within the intended patient population) was selected for further investigation in the ATTR-CM dose-expansion cohort.[34]

EX VIVO CRISPR THERAPIES

Ex vivo gene editing refers to the reprograming of specific cells via gene modification outside the human body. The desired cells are extracted, separated, enriched, and then genetically reprogrammed by insertion, deletion, or editing of a specific gene. The genetically reprogrammed cells are then allowed to undergo *ex vivo* expansion prior to patient infusion. The number of clinical investigations studying the safety and efficacy of genetically modified human cells has been growing exponentially in various therapeutic indications.[53] Currently, several genetically modified cell therapies are indicated for hematological disorders and malignancies. Earlier generations of gene-edited cells implemented the use of viral vectors to reprogram the desired cells.[54] However, the evolution of other genetic engineering tools, including CRISPR/Cas9, has expanded the effort to develop robust gene-edited cell therapies with better safety profiles and more resistance to immunosuppressive tumor environments.

Autologous vs. allogeneic cell therapy

Cell therapies are classified as autologous or allogeneic depending upon the source of the cells. Autologous cell therapy refers to the case where a patient's own cells are collected, genetically modified to express a specific protein or receptor, and then re-introduced into the same patient's body. Because the cells used for treatment are derived from the patient, there is reduced risk of immune reaction following re-infusion of the cells relative to allogeneic cell therapy, with potential enhancement of cellular kinetics (CKs).[55–57] Allogeneic cell therapy refers to the case where cells are collected from a healthy donor, genetically reprogrammed, and then used to treat a patient. Because the cells are not derived from the same patient, this increases the risk of rejection or immune reaction.

Although autologous cell therapy may pose fewer immune challenges, additional considerations can limit the effectiveness and practicality of this approach. The quality of the patient's own cells can be negatively impacted by disease burden and previous lines of treatment, which in turn may affect manufacturing success rates and both the efficacy and durability of the manufactured cells. Further, autologous cell therapy is associated with both relatively long turnaround times for individual manufacturing and processing (~ 3 weeks) and high costs for individual batch-by-batch manufacturing. The allogeneic approach presents a promising alternative to mitigate these complications. The use of relatively healthier cells as the starting material may help improve manufacturing success rates and overcome some of the limitations of autologous cells. Because the cells are pre-manufactured and utilized as "off-the-shelf" products, concerns related to long turnaround times associated with autologous cell therapy would be ameliorated. Additionally, a batch manufactured from single donor-cell material may be considered for the treatment of multiple patients using the allogeneic approach, reducing manufacturing and processing costs. The allogeneic approach simplifies patient redosing, if needed, without the need for a stored back-up batch or remanufacturing, which is not always feasible given the patient's health. These potential benefits of the allogeneic approach are potentially offset due to immunologic challenges attributed to the introduction of foreign donor cells because of HLA mismatch. Among these is graft-vs.-host disease (GvHD), which is a serious and potentially life-threatening condition where donor cells recognize the recipient cells and illicit a strong immune reaction leading to graft rejection; conversely, there is also the possibility of graft rejection via host T cells or natural killer (NK) cells.

CRISPR-based gene editing in allogeneic cell therapy

A considerable effort is underway to leverage the potential advantages offered by allogeneic cell therapy. Approaches are being studied in attempt to mitigate allogeneic cell therapy-associated immune challenges and enhance patient outcomes. Among these approaches is the implementation of gene editing tools to knock out specific genes and reduce the expression of surface proteins and receptors that initiate immune responses.[58,59] CRISPR-based gene editing offers a potential solution by enabling modifications to the genome of the donor cells before they are transplanted. This includes editing genes responsible for proteins that are recognized by the recipient's immune system as foreign or introducing genetic modifications that reduce the risk of GvHD. CRISPR has been used to knockout out β-2 microglobulin, which plays a key role in immune recognition and rejection; this reduces recognition of the donor cells as foreign by the recipient's immune system.[60–62] The GvHD risk can be reduced by knocking out genes associated with the endogenous T cell receptor (TCR) in CRISPR-modified T cells.[63] In allogeneic T cell therapies, GvHD is driven by the recognition of the recipient patient's major histocompatibility complex (MHC) antigens by TCR expressed on the donor T cell and formed by α and β subunits (αβ T cells).[64] Accordingly, knocking out αβ TCR utilizing CRISPR/Cas9 or other gene editing tools is widely investigated in the development of allogeneic T cell therapies to reduce GvHD without compromising efficacy.[64,65]

Apart from the beneficial role of applying CRISPR/Cas9 gene editing to address the immunologic challenges associated with allogeneic therapies, other applications of CRISPR technology can be used to edit the genes of chimeric antigen receptor-T (CAR-T) cells to enhance their effectiveness and safety. For example, researchers have used CRISPR/Cas9 to yield gene-disrupted allogeneic CAR-T cells deficient of PD-1 (in addition to TCR and HLA class I molecule) to make them more resistant to immune suppression by tumor cells in animal models.[61] Moreover, CRISPR technology can be used to edit T cells and other immune cells to make them better at recognizing, attacking, or exhibiting resistance to viral infections, such as human immunodeficiency virus (HIV). Allogeneic CRISPR/Cas9-edited CCR5-ablated hematopoietic stem and progenitor cells were successfully engrafted in a patient with HIV with acute lymphocytic leukemia making these cells resistant to HIV infection. Owing to a relatively low percentage of CCR5 disruption, this investigation was viewed as a proof-of-principle more so than illustration of a fully curative intervention for HIV.[66]

Cellular kinetics

Unlike conventional drugs, following cell therapy administration, the amount of drug in circulation and other body tissues is not only dependent on the administered dose.[57] Genetically reprogrammed cell therapies can undergo *in vivo* proliferation and replication to varying degrees and accordingly have been described as "living drugs."[67,68] Hence, cell therapies are not directly described by conventional PKs and ADME. CKs are commonly described in terms of distribution, expansion, contraction, and persistence phases.[57] As with conventional drugs, following i.v. administration, cell therapies exhibit a distribution phase. Unique to cell therapies, an expansion phase follows thereafter due to *in vivo* proliferation of the administered cells. For CAR-T cells, the expansion phase is followed by a biexponential contraction, resulting from rapid apoptosis and then a more gradual decline of the modified T cells that remain. Persistence refers to the duration of the detection of the cell therapy in the patient's circulation[55,56] Both expansion and persistence have been demonstrated to be important determinants of short-term and prolonged efficacy.[69,70] Although persistence has been associated with prolonged remission,[55,56,70] sustained persistence of a cell immunotherapy may result in prolonged side effects when targeting a protein that is also expressed on normal cells. For example, anti-CD19 CAR-T cell therapies were associated with prolonged B-cell aplasia, which is considered an on-target adverse event in the treatment of B-cell malignancies.[71,72] It follows that the optimal persistence required for a specific cell therapy may vary depending minimally on the nature and burden of the disease, the possibility of relapse, the potency and extent of *in vivo* proliferation of administered cells, and the extent of target expression on normal cells. As an example, the shorter persistence of allogeneic CAR-T may represent an advantage in treating indications with targets that are not highly tumor specific to reduce the likelihood of chronic toxicity.

The CKs have been reported to be dependent on various patient and disease factors, in addition to product characteristics.[73] Among these factors are the type of lymphodepletion (LD) therapy prior to CAR-T administration; patients receiving fludarabine-based

LD demonstrated longer CAR-T persistence compared to other LD regimens.[73] The inclusion of costimulatory domain (e.g., CD28) in the CAR structure resulted in prolonged persistence and enhanced antitumor activity of CAR-T cells.[74] Animal studies demonstrated that CD28 costimulatory domain in CAR-T cells yielded higher expansion and cytotoxicity, whereas 41BB was associated with longer persistence.[75]

The understanding of the impact of different gene editing approaches including CRISPR on CKs is emerging but not yet mature. For example, CRISPR-mediated knockout of endogenous TCR in donor cells, mentioned earlier as an approach to forestall GvHD, resulted in comparable expansion of edited to non-edited T-cells and superior to gene-edited cells without elimination of native TCR.[76] Also as mentioned earlier, disruption of MHC-1 mediated rejection of allogeneic cells by host T cells could be achieved via knocking out β-2 microglobulin and reducing expression of HLA-A on donor cells.[60,77] This approach can potentially enhance persistence of allogeneic cells; however, lack of HLA expression renders the cells as targets for host NK cells.[60] Several allogeneic cell therapies implementing β-2 microglobulin disruption are being studied in individual clinical trials.[3] Although a systematic, cross-study evaluation of the effect of β-2 microglobulin disruption on CKs has yet to be conducted, the allogeneic anti-CD19 CAR-T therapy CTX110 (that implements CRISPR-mediated β-2 microglobulin disruption) was reported to decline below limit of detection in patients by 3–4 weeks.[78]

Immunogenicity

Cellular and humoral immunogenicity can have an important impact on the safety, efficacy, and CKs of cell therapies.[79,80] Allogeneic cell therapies are at increased risk of developing treatment-related immune response due to the nature of being a donor-based therapy. In addition to the previously mentioned immunologic considerations (GvHD and rejection due to recognition of MHC on donor cells), there is a considerable risk of alloimmunization, which is defined as the formation of antibodies against the HLA of the donor.[58] Donor-specific antibody-mediated graft rejection has been associated with engraftment failure in organ and stem-cell transplants.[81,82] Additionally, alloimmunization may preclude re-dosing patients if needed.[58] Stimulation of the host immune system by the donor cells may also increase the risk of inflammation and cytokine release, aside from the risk of on-target cytokine release syndrome associated with cell therapies.[83–85] Multiple approaches are implemented to mitigate the risk of potential immunogenic responses associated with allogeneic therapies in current clinical investigation, including expansion of donor pools to enable increased HLA-matching or elimination of HLA on donor cells by gene editing.[59]

Translational approaches and dose selection

As with conventional drugs, there is a regulatory expectation to inform translation of cellular therapies with a robust preclinical package that can support subsequent clinical investigation.[86] As living drugs, the interaction of cell therapies with the host (i.e., the preclinical animal model and ultimately the human patient) is an important determinant of key end points, including CKs, PDs, and safety. Accordingly, clinical translation of preclinical data for gene edited cell therapies is affected both by limitations of the animal model, as well as variability in the experimental data generated in these models.[87,88] For example, immunodeficient mouse xenograft models routinely inform the preclinical pharmacology assessment for CAR-T therapies. The predicted safe and effective human dose levels based on xenograft data have routinely been biased high relative to those observed in the clinic on an mg/kg basis; this bias was in turn attributed in part to differences in tolerability across species. Given the perception of the limited predictive value of preclinical models, aspects related to starting dose and dose escalation in the FIH trial can emphasize prior clinical studies with different cell therapies.

The current challenges in informing translation in the cell therapy space has driven innovation in the application of model-informed approaches. Mechanistic modeling, especially, provides a means to scale the underlying processes governing CKs, PDs, and potentially safety to inform translation and dosing in a more robust fashion than empirical approaches that rely upon linear scaling on body size alone (i.e., inferring the biologically effective dose in humans based on observations in animal models on a per kg basis). An example of the use of a systems CK/PD model to guide translation comes from anti-B-cell maturation antigen CAR-T cell therapy idecabtagene vicleucel.[89] In this example, a stepwise approach was used to first describe *in vitro* results from co-culture experiments and then a collection of *in vivo* assessments from both xenograft models and patients from a phase I investigation in relapsed or refractory multiple myeloma. The systems PK/PD model captured several features of the CKs and response data in the clinic, as well as the steep dose–response relationship noted in this trial. This emphasis upon model performance with respect to clinical data lends credence to the application of mechanistic PK/PD modeling to guide translation. Other examples illustrate how mechanistic modeling was leveraged to inform stages of development flanking the translational space. In the preclinical space, a Shiny R-based platform called CARTmath has become available to analyze and simulate CAR-T treatment and dosing scenarios in mouse models of hematological cancers.[90] Still, another example documents how mechanistic modeling was used to capture the relationship among CAR-T dose, disease burden, and proinflammatory cytokines associated with cytokine release syndrome in patients with advanced chronic lymphocytic leukemia.[91] These additional examples reinforce the use of mechanistic modeling to inform dosing across the development spectrum for CAR-T and potentially to extrapolate to the case of *ex vivo* CRISPR therapies more broadly.

CONCLUSION

Over the last decade, advances in CRISPR/Cas systems have unleashed the promise of genome editing as a transformative, potentially curative treatment for human disease. Maximizing the clinical utility of CRISPR therapies has involved a revisit of the traditional drug development paradigm informed by previous classes of drugs. Similarly, this has necessitated a reassessment of historical approaches for clinical pharmacology and translation that have largely been informed with these previous classes of chronically administered agents. It is of note that this review has focused on systemically administered *in vivo* and *ex vivo* CRISPR

therapies. Even as important advances have been made for locally administered CRISPR therapies (e.g., EDIT-101 for blindness due to Leber congenital amaurosis 10 and administered via a subretinal injection), the emphasis on systemically administered CRISPR therapies was intended to reflect the full span of these potential considerations, as opposed to the subset that is salient only to locally administered CRISPR therapies.

Given that the first report of a systemically administered *in vivo* CRISPR therapy was published just 2 years prior to this review,[8] the current state of clinical pharmacology and translation for systemically administered CRISPR therapies in the literature is emerging and highly dynamic. Accordingly, this review is intended to provide a snapshot of the current state-of-the-art in clinical pharmacology and translation with the expectation that the field will continue to evolve as multiple CRISPR therapies advance in the clinic. An emphasis of this review has been placed on incorporating mechanistic understanding into the quantitative methodologies in support of CRISPR therapy translation and clinical development. The emphasis upon a mechanistic approach was intended to address uncertainties with application of other empirical methods that lacked historical precedent for application to CRISPR therapies, minimally given the unique aspects related to formulation, ADME, and mechanism of action that characterize systemically administered *in vivo* CRISPR therapies. Returning to the case example of NTLA-2001, a semi-mechanistic QSP approach ultimately provided robust projections of clinical PK/PD, even in the absence of any prior clinical evaluations of this particular platform. Similarly, opportunities abound to inform *ex vivo* CRISPR translation using mechanistic quantitative approaches. Given the advancement and modular nature of multiple systemically administered CRISPR therapies in the clinic, it is possible an emergent dataset may become available that can be pooled thoughtfully across multiple assets to drive both more sophisticated mechanistic modeling and empirical model development. At a minimum, it is anticipated that this expanded dataset can better inform questions related not only to central tendency, but also variability in clinical PK/PD. At most, leveraging these data within a quantitative framework can facilitate the next stages of CRISPR clinical development, including extrahepatic targeting, multiplexed targets, and corresponding greater penetrance into more disease areas.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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1. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. & Charpentier, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
2. Modell, A.E., Lim, D., Nguyen, T.M., Sreekanth, V. & Choudhary, A. CRISPR-based therapeutics: current challenges and future applications. *Trends Pharmacol. Sci.* **43**, 151–161 (2022).
3. Cerci, B., Uzay, I.A., Kara, M.K. & Dincer, P. Clinical trials and promising preclinical applications of CRISPR/Cas gene editing. *Life Sci.* **312**, 121204 (2023).
4. Katti, A., Diaz, B.J., Caragine, C.M., Sanjana, N.E. & Dow, L.E. CRISPR in cancer biology and therapy. *Nat. Rev. Cancer* **22**, 259–279 (2022).
5. Witzigmann, D., Kulkarni, J.A., Leung, J., Chen, S., Cullis, P.R. & van der Meel, R. Lipid nanoparticle technology for therapeutic gene regulation in the liver. *Adv. Drug Deliv. Rev.* **159**, 344–363 (2020).
6. Schmidt, F. & Grimm, D. CRISPR genome engineering and viral gene delivery: a case of mutual attraction. *Biotechnol. J.* **10**, 258–272 (2015).
7. Finn, J.D. *et al*. A single administration of CRISPR/Cas9 lipid nanoparticles achieves robust and persistent in vivo genome editing. *Cell Rep.* **22**, 2227–2235 (2018).
8. Gillmore, J.D. *et al*. CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. *N. Engl. J. Med.* **385**, 493–502 (2021).
9. Le Guiner, C., Moullier, P. & Arruda, V.R. Biodistribution and shedding of AAV vectors. *Methods Mol. Biol.* **807**, 339–359 (2011).
10. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research FDA guidance for industry: design and analysis of shedding studies for virus or bacteria-based gene therapy and oncolytic products. <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/design-and-analysis-shedding-studies-virus-or-bacteria-based-gene-therapy-and-oncolytic-products> (2015). Accessed June 14, 2023.
11. Chen, N., Sun, K., Chemuturi, N.V., Cho, H. & Xia, C.Q. The perspective of DMPK on recombinant adeno-associated virus-based gene therapy: past learning, current support, and future contribution. *AAPS J.* **24**, 31 (2022).
12. Zhang, X., Goel, V. & Robbie, G.J. Pharmacokinetics of patisiran, the first approved RNA interference therapy in patients with hereditary transthyretin-mediated amyloidosis. *J. Clin. Pharmacol.* **60**, 573–585 (2020).
13. Dilliard, S.A. & Siegwart, D.J. Passive, active and endogenous organ-targeted lipid and polymer nanoparticles for delivery of genetic drugs. *Nat. Rev. Mater.* **8**, 1–19 (2023).
14. International Council for Harmonisation. ICH guideline M10 on bioanalytical method validation and study sample analysis. Step 5. <https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-m10-bioanalytical-method-validation-step-5_en.pdf> (2023). Accessed June 14, 2023.
15. Tomak, A., Cesmeli, S., Hanoglu, B.D., Winkler, D. & Oksel, K.C. Nanoparticle-protein corona complex: understanding multiple interactions between environmental factors, corona formation, and biological activity. *Nanotoxicology* **15**, 1331–1357 (2021).
16. Choi, H.S. *et al*. Renal clearance of quantum dots. *Nat. Biotechnol.* **25**, 1165–1170 (2007).
17. Jain, R.K. & Stylianopoulos, T. Delivering nanomedicine to solid tumors. *Nat. Rev. Clin. Oncol.* **7**, 653–664 (2010).
18. Smith, S.A., Selby, L.I., Johnston, A.P.R. & Such, G.K. The endosomal escape of nanoparticles: toward more efficient cellular delivery. *Bioconjug. Chem.* **30**, 263–272 (2019).
19. Gertz, M.A. *et al*. Diagnosis, prognosis, and therapy of transthyretin amyloidosis. *J. Am. Coll. Cardiol.* **66**, 2451–2466 (2015).
20. Suzuki, Y. & Ishihara, H. Difference in the lipid nanoparticle technology employed in three approved siRNA (Patisiran) and mRNA (COVID-19 vaccine) drugs. *Drug Metab. Pharmacokinet.* **41**, 100424 (2021).
21. Kristen, A.V., Ajroud-Driss, S., Conceicao, I., Gorevic, P., Kyriakides, T. & Obici, L. Patisiran, an RNAi therapeutic for the

treatment of hereditary transthyretin-mediated amyloidosis. *Neurodegener. Dis. Manag.* **9**, 5–23 (2019).
22. Bisgaier, C.L., Siebenkas, M.V. & Williams, K.J. Effects of apolipoproteins A-IV and A-I on the uptake of phospholipid liposomes by hepatocytes. *J. Biol. Chem.* **264**, 862–866 (1989).
23. Akinc, A. *et al*. Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Mol. Ther.* **18**, 1357–1364 (2010).
24. Sun, K. & Liao, M.Z. Clinical pharmacology considerations on recombinant adeno-associated virus-based gene therapy. *J. Clin. Pharmacol.* **62**(suppl 2), S79–s94 (2022).
25. Maeder, M.L. & Gersbach, C.A. Genome-editing technologies for gene and cell therapy. *Mol. Ther.* **24**, 430–446 (2016).
26. Takata, M. *et al*. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *EMBO J.* **17**, 5497–5508 (1998).
27. Bae, S., Park, J. & Kim, J.S. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* **30**, 1473–1475 (2014).
28. Heigwer, F., Kerr, G. & Boutros, M. E-CRISP: fast CRISPR target site identification. *Nat. Methods* **11**, 122–123 (2014).
29. Tsai, S.Q. *et al*. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat. Biotechnol.* **33**, 187–197 (2015).
30. Cameron, P. *et al*. Mapping the genomic landscape of CRISPR-Cas9 cleavage. *Nat. Methods* **14**, 600–606 (2017).
31. Cong, L. *et al*. Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013).
32. Haussecker, D. Stacking up CRISPR against RNAi for therapeutic gene inhibition. *FEBS J.* **283**, 3249–3260 (2016).
33. Gane, E.J. *et al*. OS073–In vivo CRISPR/Cas9 editing of the TTR gene with NTLA-2001 in patients with transthyretin amyloidosis–dose selection considerations. *J. Hepatol.* **77**, S58–S59 (2022).
34. Gillmore, J.D. *et al*. First-in-human in vivo CRISPR/Cas9 editing of the TTR gene by NTLA-2001 in patients with transthyretin (ATTR) amyloidosis with cardiomyopathy. Abstract Presented at: American Heart Association Scientific Sessions 2022; November 5, 2022; Chicago, IL. <https://www.intelliatx.com/wp-content/uploads/Gillmore_NTLA-2001_ATTR_cardiomyop_AHA2022_05Nov22_vFINAL.pdf>.
35. Longhurst, H. *et al*. In vivo CRISPR/Cas9 editing of KLKB1 in patients with hereditary angioedema: a first-in-human study. *Ann. Allergy Asthma Immunol* **129**(5 suppl), S10–S11 (2022).
36. Burns, S. CRISPR/Cas9-mediated targeted gene insertion of SERPINA1 to treat alpha-1 antitrypsin deficiency. Abstract Presented at: American Society of Gene and Cell Therapy Annual Meeting; May 11, 2021; Virtual. <https://www.intelliatx.com/wp-content/uploads/ASGCT-AATD-5.11.21-Final.pdf>.
37. Sherafat, R. & AC Planning Working Group US FDA Cellular, Tissue, and Gene Therapies Advisory Committee (CTGTAC) Meeting #70: Toxicity Risks of Adeno-Associated Virus (AAV) vectors for gene therapy (GT) (Slide Deck). <www.fda.gov/media/151969/download September 2–3> (2021). Accessed June 14, 2023.
38. Mingozzi, F. & High, K.A. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood* **122**, 23–36 (2013).
39. Manno, C.S. *et al*. Successful transduction of liver in hemophilia by AAV-factor IX and limitations imposed by the host immune response. *Nat. Med.* **12**, 342–347 (2006).
40. Wright, J.F. AAV vector and manufacturing process design–how do they impact immunogenicity? Presented at FDA & ASGCT's immune responses to AAV Vectors; Virtual: January 24-25, 2023.
41. Wright, J.F. Codon modification and PAMPs in clinical AAV vectors: the tortoise or the hare? *Mol. Ther.* **28**, 701–703 (2020).
42. Mingozzi, F. *et al*. Overcoming preexisting humoral immunity to AAV using capsid decoys. *Sci. Transl. Med.* **5**, 194ra192 (2013).
43. Wright, J.F. AAV empty capsids: for better or for worse? *Mol. Ther.* **22**, 1–2 (2014).
44. Bertin, B. *et al*. Capsid-specific removal of circulating antibodies to adeno-associated virus vectors. *Sci. Rep.* **10**, 864 (2020).
45. ONPATTRO (patisiran) lipid complex injection, for intravenous use. Prescribing information. Cambridge, MA: Alnylam Pharmaceuticals; 2023.
46. Belov, A., Schultz, K., Forshee, R. & Tegenge, M.A. Opportunities and challenges for applying model-informed drug development approaches to gene therapies. *CPT Pharmacometrics Syst. Pharmacol.* **10**, 286–290 (2021).
47. U.S. Department of Health and Human Services, Food and Drug Administration, and Center for Biologics Evaluation and Research. Guidance for industry: considerations for the design of early-phase clinical trials of cellular and gene therapy products. <https://www.fda.gov/media/106369/download> (2015). Accessed June 14, 2023.
48. Tang, F., Wong, H. & Ng, C.M. Rational clinical dose selection of adeno-associated virus-mediated gene therapy based on allometric principles. *Clin. Pharmacol. Ther.* **110**, 803–807 (2021).
49. Stroh, M., Maitland, M.L., Nosbich, J. *et al*. Less may be more for TTR in ATTR: model-predicted outcomes of TTR reductions (slide deck) Presented at QSP Summit; September 15, 2022. <www.intelliatx.com/wp-content/uploads/2022-Sept-15_QSPSummit_Stroh_vf.pdf> (2022).
50. Goel, V., Gosselin, N.H., Jomphe, C., Zhang, X., Marier, J.F. & Robbie, G.J. Population pharmacokinetic-pharmacodynamic model of serum transthyretin following patisiran administration. *Nucleic Acid Ther.* **30**, 143–152 (2020).
51. McIntosh, A., Sverdlov, O., Yu, L. & Kaufmann, P. Clinical design and analysis strategies for the development of gene therapies: considerations for quantitative drug development in the age of genetic medicine. *Clin. Pharmacol. Ther.* **110**, 1207–1215 (2021).
52. Jeon, J.Y., Ayyar, V.S. & Mitra, A. Pharmacokinetic and pharmacodynamic modeling of siRNA therapeutics – a minireview. *Pharm. Res.* **39**, 1749–1759 (2022).
53. Arabi, F., Mansouri, V. & Ahmadbeigi, N. Gene therapy clinical trials, where do we go? An overview. *Biomed. Pharmacother.* **153**, 113324 (2022).
54. Vairy, S., Garcia, J.L., Teira, P. & Bittencourt, H. CTL019 (tisagenlecleucel): CAR-T therapy for relapsed and refractory B-cell acute lymphoblastic leukemia. *Drug Des. Devel. Ther.* **12**, 3885–3898 (2018).
55. Maude, S.L. *et al*. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N. Engl. J. Med.* **371**, 1507–1517 (2014).
56. Kalos, M. *et al*. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci. Transl. Med.* **3**, 95ra73 (2011).
57. Mueller, K.T. *et al*. Cellular kinetics of CTL019 in relapsed/refractory B-cell acute lymphoblastic leukemia and chronic lymphocytic leukemia. *Blood* **130**, 2317–2325 (2017).
58. Depil, S., Duchateau, P., Grupp, S.A., Mufti, G. & Poirot, L. 'Off-the-shelf' allogeneic CAR T cells: development and challenges. *Nat. Rev. Drug Discov.* **19**, 185–199 (2020).
59. Caldwell, K.J., Gottschalk, S. & Talleur, A.C. Allogeneic CAR cell therapy – more than a pipe dream. *Front. Immunol.* **11**, 618427 (2020).
60. Wang, D., Quan, Y., Yan, Q., Morales, J.E. & Wetsel, R.A. Targeted disruption of the β2-microglobulin gene minimizes the immunogenicity of human embryonic stem cells. *Stem Cells Transl. Med.* **4**, 1234–1245 (2015).
61. Ren, J., Liu, X., Fang, C., Jiang, S., June, C.H. & Zhao, Y. Multiplex genome editing to generate universal CAR T cells resistant to PD1 inhibition. *Clin. Cancer Res.* **23**, 2255–2266 (2017).
62. Kagoya, Y. *et al*. Genetic ablation of HLA class I, class II, and the T-cell receptor enables allogeneic T cells to be used for adoptive T-cell therapy. *Cancer Immunol. Res.* **8**, 926–936 (2020).
63. Manriquez-Roman, C., Siegler, E.L. & Kenderian, S.S. CRISPR takes the front seat in CART-cell development. *BioDrugs* **35**, 113–124 (2021).
64. Abdelhakim, H., Abdel-Azim, H. & Saad, A. Role of αβ T cell depletion in prevention of graft versus host disease. *Biomedicine* **5**, 35 (2017).

65. Osborn, M.J. *et al*. Evaluation of TCR gene editing achieved by TALENs, CRISPR/Cas9, and megaTAL nucleases. *Mol. Ther.* **24**, 570–581 (2016).
66. Xu, L. *et al*. CRISPR-edited stem cells in a patient with HIV and acute lymphocytic leukemia. *N. Engl. J. Med.* **381**, 1240–1247 (2019).
67. Awasthi, R. *et al*. Tisagenlecleucel cellular kinetics, dose, and immunogenicity in relation to clinical factors in relapsed/refractory DLBCL. *Blood Adv.* **4**, 560–572 (2020).
68. Tchao, N.K. & Turka, L.A. Lymphodepletion and homeostatic proliferation: implications for transplantation. *Am. J. Transplant.* **12**, 1079–1090 (2012).
69. Mueller, K.T. *et al*. Clinical pharmacology of tisagenlecleucel in B-cell acute lymphoblastic leukemia. *Clin. Cancer Res.* **24**, 6175–6184 (2018).
70. Finney, O.C. *et al*. CD19 CAR T cell product and disease attributes predict leukemia remission durability. *J. Clin. Invest.* **129**, 2123–2132 (2019).
71. Maude, S.L. *et al*. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N. Engl. J. Med.* **378**, 439–448 (2018).
72. Singh, N., Frey, N.V., Grupp, S.A. & Maude, S.L. CAR T cell therapy in acute lymphoblastic leukemia and potential for chronic lymphocytic leukemia. *Curr. Treat. Options Oncol.* **17**, 28 (2016).
73. Ramos, C.A. *et al*. Anti-CD30 CAR-T cell therapy in relapsed and refractory hodgkin lymphoma. *J. Clin. Oncol.* **38**, 3794–3804 (2020).
74. Kowolik, C.M. *et al*. CD28 Costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Res.* **66**, 10995–11004 (2006).
75. Zhao, Z. *et al*. Structural design of engineered costimulation determines tumor rejection kinetics and persistence of CAR T cells. *Cancer Cell* **28**, 415–428 (2015).
76. Prodeus, A., Yazinski, S., Dutta, I. *et al*. Engineering of highly functional and specific transgenic T cell receptor (tg-TCR) T cells using CRISPR-mediated in-locus insertion combined with endogenous TCR knockout. Poster Presented at European Society of Gene and Cell Therapy 27th Annual Meeting (2019).
77. Torikai, H. *et al*. Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. *Blood* **122**, 1341–1349 (2013).
78. CRISPR Therapeutics Updated results from the phase 1 CARBON trial of CTX110 (slide deck). <https://crisprtx.gcs-web.com/static-files/e5304031-1ceb-4db3-8451-08b1adcd3ee8> (2021). Accessed June 7, 2023.
79. Ballard, J.L., Weaver, F.A., Singla, N.K., Chapman, W.C. & Alexander, W.A. Safety and immunogenicity observations pooled from eight clinical trials of recombinant human thrombin. *J. Am. Coll. Surg.* **210**, 199–204 (2010).
80. Jawa, V. *et al*. T-cell dependent immunogenicity of protein therapeutics pre-clinical assessment and mitigation-updated consensus and review 2020. *Front. Immunol.* **11**, 1301 (2020).
81. Butler, C.L., Valenzuela, N.M., Thomas, K.A. & Reed, E.F. Not all antibodies are created equal: factors that influence antibody mediated rejection. *J. Immunol. Res.* **2017**, 7903471 (2017).
82. Ciurea, S.O. *et al*. The European Society for Blood and Marrow Transplantation (EBMT) consensus guidelines for the detection and treatment of donor-specific anti-HLA antibodies (DSA) in haploidentical hematopoietic cell transplantation. *Bone Marrow Transplant.* **53**, 521–534 (2018).
83. Fitzgerald, J.C. *et al*. Cytokine release syndrome after chimeric antigen receptor T cell therapy for acute lymphoblastic leukemia. *Crit. Care Med.* **45**, e124–e131 (2017).
84. Lee, D.W. *et al*. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet* **385**, 517–528 (2015).
85. Turtle, C.J. *et al*. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. *J. Clin. Invest.* **126**, 2123–2138 (2016).
86. US Department of Health and Human Services, Food and Drug Administration, and Center for Biologics Evaluation and Research FDA Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products. Published November 3, 2013. <www.fda.gov/media/87564/download>. Accessed June 14, 2023.
87. Abou-El-Enein, M., Angelis, A., Appelbaum, F.R. *et al*. Evidence generation and reproducibility in cell and gene therapy research: a call to action. *Mol. Ther. Methods Clin. Dev.* **22**, 11–14 (2021).
88. Duncan, B.B., Dunbar, C.E. & Ishii, K. Applying a clinical lens to animal models of CAR-T cell therapies. *Mol. Ther. Methods Clin. Dev.* **27**, 17–31 (2022).
89. Singh, A.P. *et al*. Bench-to-bedside translation of chimeric antigen receptor (CAR) T cells using a multiscale systems pharmacokinetic-pharmacodynamic model: a case study with anti-BCMA CAR-T. *CPT Pharmacometrics Syst. Pharmacol.* **10**, 362–376 (2021).
90. Barros, L.R.C., Paixão, E.A., Valli, A.M.P., Naozuka, G.T., Fassoni, A.C. & Almeida, R.C. CARTmath—a mathematical model of CAR-T immunotherapy in preclinical studies of hematological cancers. *Cancer* **13**, 2941 (2021).
91. Hardiansyah, D. & Ng, C.M. Quantitative systems pharmacology model of chimeric antigen receptor T-cell therapy. *Clin. Transl. Sci.* **12**, 343–349 (2019).
92. Zhang, Y.N., Poon, W., Tavares, A.J., McGilvray, I.D. & Chan, W.C.W. Nanoparticle-liver interactions: cellular uptake and hepatobiliary elimination. *J. Control. Release* **240**, 332–348 (2016).