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REVIEW

Manufacturing of recombinant adeno-associated viral vectors for clinical trials

Nathalie Clément[1] and Joshua C Grieger[2]

The ability to elicit robust and long-term transgene expression *in vivo* together with minimal immunogenicity and little to no toxicity are only a few features that make recombinant adeno-associated virus (rAAV) vectors ideally suited for many gene therapy applications. Successful preclinical studies have encouraged the use of rAAV for therapeutic gene transfer to patients in the clinical setting. Nevertheless, the use of rAAV in clinical trials has underscored the need for production and purification systems capable of generating large amounts of highly pure rAAV particles. To date, generating vector quantities sufficient to meet the expanding clinical demand is still a hurdle when using current production systems. In this chapter, we will provide a description of the current methods to produce clinical grade of rAAV under current good manufacturing practice (cGMP) settings.

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Significant advancements in the efficacy of gene delivery to treat human diseases using adeno-associated viral vectors (AAV), together with recent breakthroughs in manufacturing methods, has stimulated an unprecedented interest toward drug development for gene therapies and commercialization, often as a concerted and collaborative effort between academic and industry institutions.

The growing interest in AAV-based therapies lies on a series of features that make rAAV vectors ideally suited for gene transfer approaches: its ability to elicit robust and long-term transgene expression in animals and humans and its safety, demonstrated in several phase 1/2 trials, with lack of toxicity, absence of adverse events following administration and manageable immune responses.[1,2] The first AAV clinical trial was conducted in subjects with cystic fibrosis in the mid-nineties,[3] with today more than 70 approved clinical trials worldwide for a wide variety of diseases.[4] In 2012, Glybera, an AAV1-based drug for the treatment of Lipoprotein lipase deficiency in adults, was the first rAAV to be market-approved in Europe.[5] To date, no AAV drugs have been approved nor submitted for approval in the United States.

The use of rAAV in the clinical setting has underscored the urgent need for production and purification systems capable of generating very large amounts of highly pure rAAV particles. Typical FDA-approved investigational new drug include extensive preclinical studies for toxicology, safety, dose, and bio-distribution assessments, with vector requirements often reaching the 1E15 to 1E16 vector genome range. Manufacturing such amounts, although technically feasible, still represents an incredible effort when using the current production systems.

In this review, we will provide a description of the methods that have been used to produce clinical grade rAAV under current the good manufacturing practice (cGMP) settings. It is noteworthy that methods used in clinical manufacturing are often not published and therefore this review will provide general insights into the methods used to date from information gathered in detailed references or from general websites.[4,6]

TRANSFECTION METHODS

Transfection of plasmid DNA into eukaryotic cells was the first and remains the most commonly used protocol for clinical grade manufacturing of AAV. The most traditional approach is a calcium phosphate plasmid precipitation on human embryonic kidney 293 cells (HEK293)[7] or HEK293-T[8] with equimolar amount of the vector construct plasmid (prAAV-promoter-transgene) and the helper plasmid that provide the AAV Rep and Cap functions as well as the Ad5 genes (VA RNAs, E2A, and E4OEF6).[9,10] HEK293 cells constitutively express E1a/b, the fourth Ad function required for AAV replication. Cells are amplified in Corning cellstacks or roller bottles (see Table 1). A variation to this protocol is a two-helper method (or triple transfection) with AAV and Ad5 functions provided from separate plasmids.[10–13] Typically up to 80% of cells are transfected with virus production peaking between 48–72 hours in the cell harvest. The versatility of this method has been tested by the production of a variety of AAV serotypes and capsid variants including 1, 2, 2.5, 2i8, 2 triple-tyrosine mutant, 7.4, 8, 9, and rh10 for clinical trials (referenced in Table 1), as well as double-stranded AAV.[8,14]

[1]Department of Pediatrics, Powell Gene Therapy Center, University of Florida, Gainesville, Florida, USA; [2]Bamboo Therapeutics Inc., Chapel Hill, North Carolina, USA.
Correspondence: JC Grieger (jgrieger@bambootherapeutics.com)
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Table 1 Institutions and rAAV GMP manufacturing technologies

Center, location	*Production*	*Cells and platform*	*Purification*	*Serotypes*	*Removal empties*	*Reference*
Powell Gene Therapy Center, Human Applications Laboratory, University of Florida, Gainesville, FL	2-plasmid Transfection (CaPO4)	HEK293 CellSTACKS	Cell harvest microfluidization or acidic flocculation and lysis, Benzonase, Heparin AC, IEC: SP, POROS, PS, HA, Hollow fiber tangential flow filtration (TFF)	1, 2, 2(Y444, 500, 730F), 9	No	14,48,51, 56,63–70
Center for Cellular and Molecular Therapeutics, Children's Hospital of Philadelphia, PA	3 plasmid Transfection (CaPO4)	HEK293 Roller Bottles	Cell and media harvest, TFF and microfluidization, Benzonase, Poros 50HS, CsCl gradient, TFF	2	Yes	13,60, 71–77
Avigen Incorporation, Alameda, CA	3-plasmid Transfection (CaPO4)	HEK293	Cell harvest, PEG precipitation, CsCl gradient, Dialysis and concentration	2	Yes	61,78–80
St Jude Children Hospital Children's GMP, Memphis, TE	2-plasmid Transfection (CaPO4)	293-T CellSTACKS	Cell harvest microfluidization, Benzonase, Sephacryl, Poros 50HQ, Sephacryl, TFF	8	No; then Yes	8,50,81,82
Belfer Gene Therapy Core Facility, Weill Cornell Medical college, New York, NY	2-plasmid Transfection (CaPO4)	293-T CellSTACKS	Cell harvest freeze-thaws, Iodixanol, Heparin affinity, Dialysis	2, rh10	Yes	83–86
Neurologix, Ft Lee, NJ	Transfection	HEK293	Heparin affinity chromatography	2		87,88
Asklepios Bio-Pharmaceuticals and University of North Carolina, Vector Core, Chapel Hill, NC	3-plasmid Transfection (PEImax)	Suspension HEK293 Shaker flasks and WAVE Bioreactors	Cell harvest, Sonication, Benzonase, Clarification, Iodixanol gradient, Anion exchange chromatography, Diafiltration	2, 2.5, 2i8, 8, 9	Yes	15, 89–91
Harvard Gene Therapy Initiative, Boston, MA	Transfection (CaPO4)	HEK293 CF-10 flasks	Freeze/thaw, Benzonase, Detergent, Iodixanol, Chromatography	1, 2		92
Ceregene Inc, San Diego, CA	Transfection (CaPO4)	HEK293	Heparin Affinity	2	No	93
Targeted Genetics Corporation, Seattle, WA	wtAd5 Infection	HEK293 Producer HeLa Cell line WAVE and stir Tank bioreactors	Depth filtration, Benzonase, Ion exchange, UF/TFF, Chrom step, Heat inactivation, Chrom step, NanofiltrationPolishingFormulation	1, 2		94
AGTC, FL at SAFC, CA	HSV Infection	sBHK WAVE bioreactors	Detergent lysis, Benzonase, Depth filtration, TFF, Ion Exchange chromatography, Affinity chromatography, TFF	1	No	38,41,42, 95,96
UniQure/AMT	Baculovirus Infection	Sf9 WAVE bioreactors	Proprietary	1		5

The information in the table was assembled utilizing information gathered in detailed references or from general websites.

The main limitation to the transfection method of adherent HEK293 cells is its inherent lack of scalability. Scaling-up a method relying on adherent cells would require the linear multiplication of the number of flasks/surface area needed. A typical GMP manufacturing campaign requires more than a hundred cellstacks for yields often lower than 1E15 vg of clinical product (Clement N, unpublished data).[8] Based on these numbers, generating >1E16 vg would require greater than 500 cell factories. Although technically possible, it is likely not a viable option for most if not all manufacturing facilities with regard to the man power, space, and cost limitations.

An attractive option to further expand the capacity of vector production using transfection is the cell suspension format. Grieger *et al*. at the University of North Carolina Gene Therapy Center Vector Core developed a scalable, transfection-based manufacturing process utilizing suspension HEK293 cells. HEK293 cells from a qualified clinical master cell bank were adapted to grow in suspension in an animal component and antibiotic-free media, in shaker flasks, WAVE bioreactors and stir tank bioreactors. Yields generated upon triple transfection with PEImax, are typically greater than 10^5 vector genome containing particles vg/cell in crude lysates or greater than 1×10^{14} vg/l of cell culture (1×10^6 cells/ml) after 48 hours incubation.[15] Parameters including selection of a compatible serum-free, chemically defined suspension media optimal for both cell growth and transfection, selection of a transfection reagent, transfection conditions and cell density, were fully optimized. Early process development data supports a linear increase in rAAV yields with respect to cell density, achieving prepurification yields of ~1×10^{15} vg/l of high cell density cell culture in both shaker flasks and small scale stir tank bioreactors to be scaled to 250L single-use bioreactors (Grieger JC, unpublished data). Increased yield recovery using improved purification methods will achieve >4×10^{14} purified AAV vector per L of transfected high cell density culture (Grieger JC, unpublished data). This platform has all the prerequisites to enable rapid and scalable rAAV production for larger-scale manufacturing campaigns.

The overall advantages of the transfection method are: simple protocols, ease and relatively low cost to generate and test the raw materials

(plasmid preparations) due to current technologies available to manufacture high plasmid yields (0.5–1 g/l),[2] versatility to generate different rAAV constructs and serotypes in a relatively short turn-around time, which is ideal as no need to generate and characterize several cells clones (MCBs and WCBs) and viral banks (MVBs and WVBs for Ad, Bac or herpes simplex virus (HSV)), high particle yield per cell in crude lysates (>10^5 vg/cell) with optimized protocols, standard physical-to-infectious particle ratios and the largest portfolio of historical data pertaining to clinical safety. Most of the current rAAV GMP manufacturing campaigns for use in phase 1/2 human gene therapy clinical trials have been generated using transfection and are summarized in Table 1.

CLINICAL MANUFACTURING USING PACKAGING AND PRODUCER CELL LINES

Stable cell lines, usually derived from HeLa cells, are engineered by introducing either the AAV *rep* and *cap* genes (packaging cell lines) and/or the rAAV genome to be produced (producer cells).[16–18] One interesting feature of packaging and producer cell-based production relates to their ability to generate relatively high rAAV particle-per-cell yield. Yields as high as 10^5 vg/cell have been reported with vg/infectious unit (i.u) ratio comparable to transfection-produced rAAV. rAAV is produced from packaging cell lines upon transfection of the AAV construct and coinfection with an Ad helper virus, or alternatively, upon infection with a recombinant Ad/AAV hybrid helper. For producer cells containing both AAV rep/cap and rAAV genome, production is a single-step infection with an Ad helper virus.[19,20] Several AAV clinical lots were produced using HeLa producer cell lines with Ad or Ad/AAV infection (Table 1), including tgAAVCF for cystic fibrosis (HeLa JL L-14), tgAAV94 for arthritis (HeLa 51-H5), and an AAV2 (α, β, γ, or Δ) for limb girdle muscular dystrophy (HeLa B50).[21] Clinical data reported no adverse event related to the AAV product.[22] A more scalable approach takes advantage of the HeLaS3 cell line, a HeLa derivative capable of growing in both adherent and suspension formats. The cell line was engineered using traditional stable transformation procedures in flasks, and the large-scale manufacturing can occur in suspension format in spinner or bioreactors. Mydicar (AAV1), a product for heart failure, was produced using HeLaS3 producer cell line 7H11-11D3 upon infection with a defective E1-deleted Ad5/AAV hybrid helper and Ad5 at a 2000L scale.[23,24]

Three drawbacks have limited the use of stable cell lines in the clinical setting. First, the generation of stable cell lines is usually cumbersome and needs to be established for each vector and serotype combination. Second, characterization and stability of the stable cell lines could be cumbersome including risks related to the negative influence of passage history on growth kinetics and vector production capabilities. Another concern is the use of Ad5 or variants and establishing downstream purification procedures to remove wtAd5 from the rAAV product as an MOI of 100 wtAd5 per producer cell in culture has been reported to generate higher yields of rAAV.[19]

CLINICAL MANUFACTURING USING THE BACULOVIRUS PRODUCTION SYSTEM

To this date, the only clinical AAV produced using the BV system is the AAV1-based drug Glybera. It is also the only AAV product currently marketed. Procedures to manufacture Glybera have not been published in detail. It is known however that the BV system derived from Dr. Kotin's earlier work was used.[25] This system, as well as recent improvements will be discussed below.

Recombinant baculovirus derived from the *Autographa californica* nuclear polyhedrosis virus has been widely employed for large-scale production of heterologous proteins in cultured insect cells for several reasons:[1] high-level expression of foreign proteins;[2] insect cells grow in suspension culture at high cell densities and[3] post-translational modifications of the heterologous proteins are typically similar to the those in mammalian cells generating biologically active molecules. The BV system was employed to generate AAV-like particles[26] and full rAAV2 vector preparations.[25] The latest work paved the way to the commonly used systems and demonstrated that viral helper functions from Adenovirus or Herpesvirus were not needed. AAV2 was produced in Sf9 insect cells upon coinfection with three recombinant baculovirus vectors, encoding the rep gene, the cap gene, and the rAAV genome.[27] This original design was further simplified to a two BV system by combining the rep and cap expression cassettes.[28] Further, Cecchini and colleagues developed a platform using single aliquots cryopreserved baculo-infected insect cells for rAAV production to circumvent BV inherent loss of infectivity over time. The BV-infected cells with each of the AAV components (AAV and Rep/cap) are used to inoculate the bioreactor in the presence of insect cells, releasing infectious rBV on a continuous basis during the production phase.[29] This design was successfully scaled-up to 200L scale in bioreactors and resulted in yields above 1E16 vg of purified material, with an average of ~2E4 vg/cell,[29] in a highly reproducible manner.

The early developmental work raised a series of issues pertaining to BV stability upon amplification, resulting in decreased rAAV yields, as well as required modification to the AAV capsid genes to restore infectivity of several serotypes.[30] To improve the system stability and overall efficacy and simplicity, Aslanidi *et al*.[31] developed a two-component production system with the engineering of SF9 stable cell lines containing integrated copies of AAV rep and cap. BV regulatory elements were added to control AAV Rep and Cap expression in an inducible manner. AAV production is triggered with a single rBV infection containing the rAAV genome. This strategy has now been employed to generate AAV stocks for serotype 1 to 12.[32]

Several drawbacks seem to have limited the use of the Baculovirus system to produce AAV vectors for use in the clinical setting. These drawbacks have been related to a molecular/cellular biological learning curve spanning greater than a decade to understand the nuances of assembling a fully functional mammalian rAAV virus in insect cells using insect viruses. Some of these drawbacks included: instability of the AAV cassette in baculoviruses during the expansion phase to high titers,[30] inability to generate/assemble infectious AAV particles with correct ratio of capsid proteins[26,30,33,34] and inefficient viral clearance strategies to remove input and propagated baculoviruses from AAV through purification methods (patent WO 2013036118 A1).

Despite these limitations, recent improvements have demonstrated the capability of the system to produce large amounts of rAAV vectors in a scalable manner. Several biopharmaceutical companies are currently using this platform to support large-scale clinical rAAV manufacturing in the next few years.

CLINICAL MANUFACTURING USING THE HERPES SIMPLEX TYPE I SYSTEM

The first clinical trial conducted with an AAV1 manufactured using recombinant HSVs was launched in 2010 for α1-antitrypsin deficiency.[35] Like adenovirus, HSV type I is a well-described helper virus for AAV that creates a favorable environment for AAV replication in mammalian cells. The current system, created in the late nineties,[36,37] utilizes two engineered recombinant HSVs, carrying either the rAAV

vector genome containing the expression cassette with the therapeutic gene, or the AAV rep/cap packaging functions. AAV production is triggered by the simultaneous infection of HEK293 or BHK with both rHSV stocks at optimized multiplicity of infection (MOIs), in an adherent or suspension format (reviewed in ref. 38). For safety and yield optimization, rHSV are built on the replication-deficient d27.1 HSV variant, lacking ICP27 expression. For this reason, viral banks are produced in V27 cells, a VERO-derived cell line that supports rHSV replication.

Recent improvements have enabled the rHSV system to be fully compatible with large-scale rAAV production requirements for clinical manufacturing leading to the first trial.[39] The major improvement was the use of suspension-adapted Baby Hamster Kidney cells (sBHK) to enable AAV production in WAVE bioreactors at a > 100L scale.[39,40] In addition to enable linear scale-up of the production platform, the suspension format featured intrinsic advantages including a higher cell density, resulting in higher number of producer cells per volumetric unit, and reduced MOIs used to produce similar amounts of AAV.[39] Further, the system showed suitability to produce a variety of constructs and serotypes, including AAV1, 2, 5, 8, and 9 (Clement N, unpublished data).[39] Scale-up was also promoted by the use of micro-carriers to produce rHSV working banks in a suspension format in bioreactors.[41]

This system enabled particle yields per cell largely superior to 1E5 vg/cell[39,40] (Clement to be published) in crude lysates. The 100L production campaign for the clinical AAV1 generated >1E16 AAV vg in the crude harvest (~1–2E11 cells). However loss during the purification processes resulted in a final yield of about 2E4 vg/cell of purified vector.[40] Interestingly, the biological characteristics of the HSV-generated AAV1 were very favorable: increased potency, as noted by lower ratios of vector genome unit to infectious units as well as increased expression of the therapeutic gene upon cell transduction, and overall reduced amount of empty capsids. (Clement N, unpublished data).[40] The clinical product met all release criteria pertaining to safety, with no adverse events reported and near complete HSV clearance was documented.[42] Building on these pivotal findings, several constructs are being developed using this method (Clement N, unpublished data). The system is currently being adapted to serum-free media (Knop DR, unpublished data). Final yields > 1E5 vg/cell of purified vector could be obtained for other serotypes using different purification processes (Clement N, unpublished data).

Limitations to the system and anticipated challenges for clinical use are similar to those described earlier for the baculovirus system with a relatively more complex preparation of the upstream reagents (rHSV and banks), and purification processes adapted to ensure complete removal of HSV-derived products and associated detection assays.

PURIFICATION METHODS FOR CLINICAL MANUFACTURING

The overall success of rAAV clinical manufacturing critically relies on downstream purification steps with the ultimate goal of generating a final clinical product of high titer, high potency, and high purity. Processes ensuring the highest recovery and consistency, and suitable for future marketing large-scale requirements will be selected. rAAV purification faces several challenges: (i) separation of rAAV particles from cellular and viral impurities (nucleic acids and proteins) is tailored to each capsid serotype, (ii) removal of AAV empty capsids still relies on cumbersome density gradients, and (iii) potency and/or stability of the purified particles may vary according to the methods used for production, purification, and final formulation buffer.

Since the AAV clinical manufacturing field is still in an early stage, methods are often being developed for each new product in each isolated group, creating a large portfolio of methods and lack of standardization. Purification processes occur at least in five major phases: (i) harvest of the producer cells, occasionally with the supernatant, (ii) chemical (detergent and ion containing lysis buffers) and/or mechanical (freeze-thaw, microfluidization) cell lysis to liberate AAV particles, (iii) cellular and viral nucleic acid removal by enzymatic digestion (Benzonase), (iv) one to three step particle separation by chromatography and optionally density gradients, and (v) concentration, formulation, and sterile filtration (Table 1).

Typically, AAV is mostly present in the producing cells and traditional spin centrifugation is used to collect the cells and discard the supernatant (Table 1). There has been some indication that depending on the production method, timing of harvest, and/or the serotype produced, a significant amount of viral particles may also be purified from the supernatant (Table 1). *In situ* lysis followed by filtration and concentration by tangential flow filtration are used to clarify the whole cell lysate and to reduce the working volume from liters of production.[40] This approach poses challenges: the sample volumes to be processed require adapted equipment and space, increase processing times, added processing steps and increase the cellular, serum and viral (when using infection) burden through the subsequent steps. Convincing evidence that the gain in vector load from the supernatant out-weighs the disadvantages of processing several hundred liters of samples remain to be further evaluated.

Affinity and ion-exchange chromatography (IEC) methods are widely utilized for AAV separation due to their efficiency, versatility, and scalability potentials. Affinity chromatography relies on the attachment of the viral particle to a specific substrate that mimics the genuine cellular receptor while IEC separates charged molecules based on electrostatic interaction between the molecules, in this case AAV capsids, and the ionized groups incorporated into the column matrix. In both cases, matrix-bound molecules are commonly eluted by increasing the ionic strength through an increase in salt concentration. AAV2 is the only serotype in the clinic purified over heparin affinity chromatography.[7,10,43–47]

Conversely, ion-exchange chromatography is in theory applicable to all AAV serotypes or capsid variants. However, the physicochemical property differences across AAV serotypes entails individual optimization. Prepacked columns and or resins are chosen based on their quality, reliability, notably consistency across lots, and when possible their scalability. Cleaning-in-place procedures will ensure sterility of the product, and allow purification of multiple batches of the same product, if desirable. Complete change-over procedures, with replacement of all the equipment parts that have been in direct contact with the product, must be performed between campaigns for different products. To date, serotypes 1, 2, 8, 9, rh10 and variants (Table 1) have been successfully purified using one or two-step IEC protocol.[7,13,28,39,48–58]

A potential drawback of IEC is the difficulty to separate empty from full particles and no protocols have yet been implemented for the clinical setting.[54,59] To date, the only method used to remove empties from clinical AAV stocks is density gradient based on either iodixanol[15] or cesium chloride[60] (Table 1). The gradient separation occurs at early stage in the purification process, depending on the total sample volume, or at a later point (post IEC) as a polishing step.[13,61] Disadvantages of this approach are lengthy processing time, risk of vector contamination during these open steps, the need to remove and test for trace of iodixanol or cesium chloride, and its lack of scalability.

Purification processes are often completed by a concentration and/or buffer exchange step for final formulation. Tangential flow filtration is the most widely used technique and separation is based on size exclusion.

A universal purification strategy (Table 1) was developed by Grieger *et al*.[15] that results in high purity vector preps for all AAV serotypes and various chimeric capsids tested, high full to empty particle ratios (>90% full particles) and provides postpurification yields (>1×10^{13} vg/l) and purity suitable for clinical applications (meets all release criteria).

RELEASE TESTING OF CLINICAL RAAV FINAL PRODUCTS AND INTERMEDIATES

Release testing of final product is dictated by a series of FDA-established requirements and predetermined specifications to determine safety, purity, concentration, identity, potency, and stability of the product.[13] Safety assessments include product contaminants and product-related impurities. Characterization of the final product includes a determination of the viral genome titer, infectious titer (potency), product identity (AAV capsid and genome), and therapeutic gene identity (expression/activity). GMP-compliant in-house assays are submitted to a qualification process to define linearity, reproducibility, specificity, sensitivity, robustness, and the range of internal controls. An independent quality assurance unit reviews both manufacturing and QC processes for compliance with the stated guidelines and regulations. Product stability is evaluated at predetermined time points and for the duration of the clinical trial. Infection-based methods, including Adenovirus, Herpes virus, and Baculovirus, require additional testing related to the helper system derived contaminants and impurities.[40,62]

The lack of standardized QC methods prevents a thorough and unbiased comparison of the current investigational new drugs related to production yields (vector genomes), product potency (infectious particles and product expression), capsid load, ratio of full/empties, and biochemical purity is described in detail in Wright *et al*.[13]

A thorough evaluation of a viral clearance step for rAAV produced by infection is essential for a critical path toward marketing and establishing safety of bio-drugs. Viral clearance studies are prerequisite to assess process steps efficient at removing viruses, including replication competent viruses that may arise during the production process, and follow guidance from FDA and International conference on harmonization (ICH).

CONCLUSION

Safety and in some cases efficacy of the rAAV vector system has been demonstrated in more than 70 clinical trials initiated using current technologies. Significant advances as well as their respective limitations of each manufacturing method have been highlighted in this review. Efforts must now focus on robust production scale-up platforms to transition to industry for late phase clinical trials and commercialization, as well as method standardization related to both manufacturing and quality control. As the race continues for yields beyond 1×10^{17} vg, the overall vector quality and potency must be the intrinsic and most determining values to assess, as it will directly affect the overall therapeutic efficacy and safety pertaining to transduction ability and immune response management.

Methods described in this review all have advantages and disadvantages and many are still in the preliminary evaluation stages. Collaborative and unbiased preclinical and clinical studies, both at academic and industry levels, and possibly guided by the FDA or committees of expert scientists, will be critical in assessing each method's true capability and building the foundations of future methods from lessons learned from each.

CONFLICT OF INTEREST

J.C.G. is a cofounder of Bamboo Therapeutics, Inc., holds patents that have been licensed by University of North Carolina to Asklepios Biopharmaceutical, for which he receives royalties. He has consulted for Asklepios Biopharmaceutical and has received payment for consultation.

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