# CAR T-CELL THERAPY AS AN INNOVATIVE APPROACH IN CANCER IMMUNOTHERAPY

## TERAPIA CAR T JAKO NOWOCZESNA STRATEGIA W IMMUNOTERAPII NOWOTWORÓW ZŁOŚLIWYCH

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*Summary:* Over the recent years, immunotherapy has gained the reputation of an modern and effective, molecular targeted method of cancer treatment. The cancer immunotherapy includes stimulation of the immune system to selective, anti-tumor response performed by lymphoidal lineages of blood cells. This strategy is used in the innovative *CAR T-cells therapy* (*chimeric antigen receptor T-cell therapy*) which uses T lymphocytes to specific immune response against surface antigens of tumor cells.

The mechanism of CAR T-cells therapy involves in the genetic modification of autologous or allogenic T lymphocytes, enabling the expression of a *chimeric antigen receptor* (CAR). CAR should be specific for an antigen located on the surface of tumor cells. Genetic material can be introduced into T lymphocytes using viral vectors, DNA transposons or by electroporation as a transcribed mRNA fragment. CAR receptors enable T lymphocytes to recognize the cancer and the activation of the anti-cancer reaction. CAR T-cells act independently of the major histocompatibility complex (MHC). Modified *in vitro* and multiplied by the hundreds of millions, T-lymphocytes are then infused into the patient's blood, where they participate in a specific, anti-cancer immune response.

In 2017, CAR T-cell therapy was approved by the *Food and Drug Administration* for clinical use in the treatment of acute lymphoblastic leukemia and B-cell non-Hodgkin's lymphoma. Since then, a rapid increase of research interest in the CAR T method has been observed. Numerous studies are underway to introduce the CAR T-cell therapy to the treatment of not only hematological malignancies, but also solid tumors. The aim of the following work is the summary of current knowledge about CAR T-cell therapy, with particular emphasis on the methods of T-cells engineering and the potential use of therapy in medicine.

*Keywords:* chimeric antigen receptor, immunotherapy, CAR T-cell therapy, pancreatic cancer

*Streszczenie:* W ciągu ostatnich lat immunoterapia zyskała opinię nowoczesnej i skutecznej, ukierunkowanej molekularnie metody leczenia nowotworów. Do metod immunoterapii nowotworów zalicza się między innymi stymulację układu odpornościowego do selektywnej odpowiedzi przeciwnowotworowej realizowanej przez komórki linii limfoidalnej. Strategia ta wykorzystywana jest w innowacyjnej terapii CAR T (ang. *chimeric antigen receptor T-cell therapy*) wykorzystującej limfocyty T do swoistej odpowiedzi odpornościowej skierowanej przeciwko antygenom powierzchniowym komórek nowotworowych. Terapia CAR T polega na genetycznej modyfikacji autologicznych lub allogenicznych limfocytów T, umożliwiającej ekspresję na ich powierzchni *chimerycznego antygenu receptora (CAR)* – białka specyficznego dla konkretnego antygenu zlokalizowanego na powierzchni komórek nowotworowych. Materiał genetyczny może zostać wprowadzony do limfocytów T z użyciem wektorów wirusowych, transpozonów DNA lub metodą elektroporacji jako przepisany fragment mRNA. Receptory CAR nadają limfocytom T zdolność rozpoznawania nowotworu, a w efekcie aktywują reakcję przeciwnowotworową. Powstałe w ten sposób *komórki CAR T* działają niezależnie od antygenów zgodności tkankowej MHC. Zmodyfikowane *in vitro* i namnożone w liczbie setek milionów, limfocyty T są następnie poddawane reinfuzji do organizmu pacjenta, gdzie uczestniczą w swoistej, przeciwnowotworowej odpowiedzi immunologicznej.
W roku 2017 terapia CAR-T została zaakceptowana przez *Food and Drug Administration* do użytku klinicznego w leczeniu ostrej białaczki limfoblastycznej oraz chłoniaków nieziarniczych z dużych komórek B. Od tej pory obserwuje gwałtowny wzrost zainteresowania metodą CAR T. Trwają liczne badania nad zastosowaniem metody nie tylko w leczeniu nowotworów układu krwiotwórczego, ale również guzów litych. Niniejsza praca ma na celu podsumowanie obecnej wiedzy na temat mechanizmów działania technologii CAR T, ze szczególnym uwzględnieniem metod modyfikacji limfocytów T oraz potencjalnego zastosowania terapii w medycynie.

*Słowa kluczowe:* terapia CAR T, immunoterapia, rak trzustki

*List of abbreviations:* **ALL** – acute lymphoblastic leukemia; **CLL** – chronic lymphocytic leukemia; **CRS** – cytokine release syndrome; **DLBCL** – diffuse large B-cell lymphoma; **EMA** – European Medicines Agency; **FDA** – U.S. Food and Drug Administration; **i.v.** – intravenous; **MHC** – major histocompatibility complex; **PMBCL** – primary mediastinal large B-cell lymphoma; **R/R** – relapse and refractory; **scFv** – single chain variable fragment; **TAA** – tumor associated antigen; **TCR** – T cell receptor; **TM** – transmembrane domain; **TME** – tumor microenvironment

## INTRODUCTION

For decades, the cancer treatment has been based on surgery, chemotherapy and radiotherapy. However, these conventional approaches for neoplastic diseases present reduced efficacy in the treatment of cancer at advanced stages. The development of oncology over the last years allowed to introduce new class of treatment options – *biological therapy*, which includes immunotherapy, gene therapy and other molecular targeted therapies.

Immunotherapy revolutionized the field of oncology as the group of methods enhancing and augmenting the natural function of the immune system to fight

cancer [22]. In physiological conditions, immune mechanisms are involved in the identification and elimination of incorrectly multiplying cells, and thus prevention of tumor formation. The prime anticancer activity is attributed to natural killer (NK) cells, lymphocytes T, macrophages and dendritic cells [10]. The imbalance between immune system activity and recognition of tumor cells enables the carcinogenesis and proceeds further growth of cancer.

Immunotherapy can increase the cancer cells "detectability" to the immune system. An innovative immunotherapeutic approach called "*chimeric antigen receptor T-cell therapy*" (CAR T-cells therapy) is based on the collecting and using a patients' own T-cells to treat their cancer. *Chimeric antigen receptors* (CARs) are receptor fusion proteins designed to predispose T-cells to target a specific antigen protein localized on the extracellular domains of cancer cell's membrane (*tumor-associated antigen* – TAA). The function of CARs is to bind the cancerous antigen and activate T-cells. Therefore, the mechanism of CAR T-cell therapy assumes the usage of T-cells engineered with CARs for anticancer treatment [28].

The first chimeric receptor was designed and described in 1989 by Eshhar et al. from the Weizmann Institute of Science in Israel [13]. Since then, many clinical trials have been performed in order to develop method and improve its effectiveness against particular types of cancer. Nowadays, CAR T-cell therapy is gaining more and more interest. In 2017 *Food and Drug Administration* (FDA) approved two CAR T-cell protocols for the treatment of acute lymphoblastic leukemia (ALL) in children and diffuse large B-cell lymphoma (DLBCL) in adults [32]. In 2018 *European Medicines Agency* accepted CAR T-cells therapy for the clinical practice as well [48]. The further studies are being conducted – both on hematological and solid malignancies, to widen the application of the method in clinical practice [55].

The following review presents the current knowledge on CAR T-cells engineering, as well as their application options in the treatment of blood cancers and solid tumors.

## CAR T-CELLS ENGINEERING

CAR consists of three main domains: extracellular, transmembrane and intracellular [14]. The extracellular domain is made of either single chain variable fragment (scFv) derived from monoclonal antibody or fragment binding an antigen (Fab) and a spacer element. The leading function of scFv is recognizing and binding to antigens expressed on the targeted cell surface tumor-associated antigens (TAAs) [14]. The process takes place by bypassing antigen presentation involving MHC, which allows for the direct recognition of the tumor cell. The spacer element is assembled of the linker between the Fc part and Fab arms, mostly derived of IgG1, also called hinge region. Its function is to improve stability, binding and signaling [31]. More-

over, flexibility of obtain the antigen depends on the length of hinge region. Spacer connects the transmembrane domain (TM) with the antigen binding domain and intracellular signaling part created of γ – chain from FcεRIγ or ζ -chain from the TCR complex. The TM domain consists of membrane protein such as CD28, CD8, CD4, FcεRIγ or CD3ζ [31, 49]. TM domain function is anchoring CAR in the membrane of T-cell and signaling. There are three generations of CAR-T cells depending on the number of intracellular domain structures transmitting signals (fig. 1):

- First generation CARs contained only the CD3ζ (ζ chain of complex TCR/CD3).
- Second generation CARs beside CD3ζ hold CD28 or 4-1BB sequence from a co-stimulatory molecule promoting synthesis of IL-2 to avoid apoptosis and activate T cells.
- Third generation CARs are characterized by combing CD3ζ with two multiple co-stimulatory signals to enhance cytokine production, proliferation and killing ability. These could be the following sequences of CD27, CD28, CD134 (OX40) CD137 (4-1BB) or DAP10 [14].

## GENETIC MATERIAL INTRODUCTION

The high efficiency and stable transgene expression is a desirable effect on genetic material introduction. There are several methods of viral and non-viral gene delivery with their advantages and disadvantages including: viral transduction,

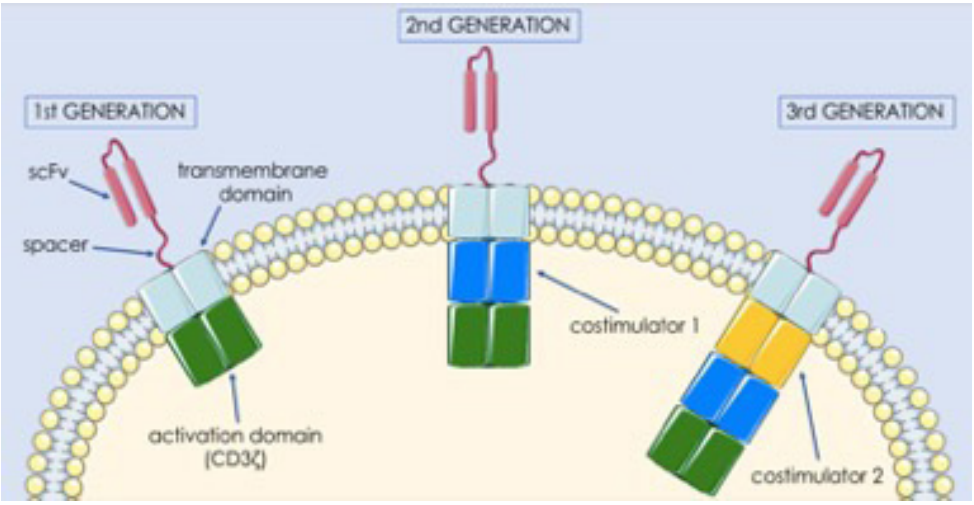


**FIGURE 1.** Schematic structure of three generations CARs (based on [54] and Servier Medical Art website, http://smart.servier.com)
**RYCINA 1.** Schematyczny obraz trzech generacji chimerycznych receptorów antygenowych (na podstawie [54] i strony internetowej Servier Medical Art: http://smart.servier.com)

transposons, CRISPR/Cas9, electroporation and other non-viral transfection like liposomes. For the purpose of CAR-T cell therapy the most commonly used are viral transductions and electro-transfection [28].

## VIRAL TRANSDUCTION

Viral transductions are the most frequently utilized to modify T-cells with chimeric antigen receptors based on the infectious virus structure, including retroviruses, adenovirus or adeno-associated virus [40, 51]. The principal advantages of viral systems are high transduction efficiency (30%-80%) or integrating into the host genome triggering permanent transgene expression with comparative simplicity of manufacturing and production [51]. For clinical approaches viral delivery platforms should have low immunogenicity and genotoxicity with the replication inability [28].

Moloney murine leukemia virus (Mo-MLV), simple γ-retroviruses were used as a successful engineered pioneers for the gene transfer. Up to now, lentiviral vectors based on HIV are the most frequently used [30]. Genes such as *pol, gag, env* or *rev* for lentiviruses do not occur in viral backbone. They are provided in helper plasmid and the CAR transgene is placed in their position. During the procedure of packing cells are transfected with helper plasmid and CAR transgene vector, generating pseudoviral particles, stable produced in cell lines. T-cells are activated with OKT3/CD28 beads and incubated with particles for integration into the host genome and gene expression [28].

The main obstacle of viral transduction for CAR-T cell approach is still relatively high costs of manufacture [16].

## NON-VIRAL TRANSFER METHODS

Non-viral methods of gene delivery, like plasmid DNA or IVT mRNA introduction are safe, simple to use and produce [17]. Moreover, these methods have low risk of mutagenesis and immunogenicity [28].

Unfortunately, these transfer techniques are limited for their time of transgene expression due to dilution or/and degradation of the vector, cellular toxicity and ineffective cellular delivery [17]. Delivering mRNA has also some obstacles. mRNA has negative charge and is susceptible to degeneration, sensitive, instable, with too low translation efficacy and has immunostimulatory effect. An enhanced translation efficiency can be gained by modification of mRNA structure such as addition of ARCAs (anti-reverse cap analogs), poly(A) tail, replacement of AREs (adenylate-uridylate rice) sequence with 5'UTR or changing 3'UTR AREs with β-globin. mRNA does not need to reach nucleus expressing protein in the cytoplasm [28].

DNA and mRNA non-viral delivery can be achieved by electroporation, gene gun, transposons, CRISPR/Cas9 or by nanoparticles (viromers, protamine-mRNA complexes, polymeric, lipid or gold nanoparticles etc.) transported by endocytosis [26].

Lipofectamine reagents, made of cationic lipids are commonly used to transfer mRNA or DNA into eukaryotic cells [7]. It is possible by influence of phosphate groups in the nucleic chain with liposomes, which are positively charged allowing fusion with membrane [28].

More promising, faster and cheaper approach is electroporation [41]. Gene electrotransfer is an efficient, faster and in theory safe nonviral method for the CAR IVT-mRNA delivery of all sizes and does not cause transgene integration to host genome [20]. This physical process causes temporally permeabilization of cells between two electrodes, which allows to intake the genetic material [30]. However the effectiveness of electroporation for gene transfer depends on electrical properties (number, shape and length of pulses, electric field intensity and frequency) number of cells and concentration of plasmid [51].

### Transposons

Transposons are dual mobile DNA sequence able to integrate transgene to host genome in the stable manner. For gene transfer delivery approach transposons are composed of 2 separated plasmids. First carries the CAR transposon and the second the transposase [28, 30]. Transposase flanks sequence of CAR and acts on ITRs (inverted terminal repeats) triggering cutting, removing and consecutive integration at the target cell genome. Both plasmids can be electrotransfered to T-cells [25]. After genomic integration, chimeric antigen receptors are expressed on the T-cell surface.

There are at least two transposon-based gene delivery systems Sleeping Beauty (SB) and piggyback [30]. The first transposon able to efficient transposition was SB, till now this is the most advanced system for CAR T cell engineering. This method combines benefits form viral vectors with favorable features of naked DNA sequences [17]. Piggyback system has greater transfection efficiency and is able to transpose larger gene compare to SB [28].

Main advantages of using transposons in CAR therapy are higher effectiveness, longer duration of transgene expression in vivo and ex vivo, lower costs of manufacturing and lesser toxicity compare with traditional plasmids or viral vectors [16, 17].

## CRISPR/CAS9

In CAR-T cell therapy gene editing methods such as clustered regularly interspaced short palindromic repeat (CRISPR) are used to knock out gene of interests in the genome e.g. TCR receptor to prevent GvHD (graft-versus-host disease) or to avoid rejection of modify T-cells [45]. CRISPR/Cas9 technology can be also used to modify and produce CAR T-cells with high homogeneity and survival rate. The crucial idea for this method is controlled CAR's integration and elimination of unpredicted integration of viral transduction. Here, endonuclease, Cas9 can be delivered through electroporation [45], using liposomes, chemical compounds, nanomaterials like biotin-streptavidin conjugate or via viral transduction [28].

## BINDING AN ANTIGEN BY CHIMERIC RECEPTORS

Engineered T-cells bind the cancer-surface antigens with MHC-independent manner with scFv. Antigen displayed on the cancer cell surface is not presented with major histocompatibility complex. It allows the activity of the same CAR molecule in both $CD4^+$ and $CD8^+$ T cells [18]. After antigen binding, the intracellular domain transmits an activation signal. T-cell activation is expected in mediation through highly organized complexes – *immunological synapses* (IS). A matured IS is an aggregation of T-cell receptor-based signalosomes inducing T-cell responses [43]. The chimeric pairing of an antigen receptor with engineered CAR and following intracellular signaling cascade allows T-cells to target cancer cell epitopes independent on the major histocompatibility complex.

## APPLICATION OF CAR T-CELL THERAPY

### CAR T-CELLS AGAINST HEMATOLOGICAL MALIGNANCIES

Therapy with CAR-expressing T-cells has emerged as one of the most promising immunotherapeutic approach, particularly in the treatment of blood cancers. The best results has been observed, while using anti-CD19 CAR T-cells. CD19 is a transmembrane protein widely expressed during B-cell lymphopoiesis on all normal B-cells and malignant B-cells as well [39]. CAR T-cell therapy targeted against CD19 is based on CD19-specific chimeric antigen receptors recognizing antigens expressed on CD19+ B-cells. CD19 induces signaling cascade, which leads to T-cells activation and proliferation, cytokine production and, as a result, lysis of targeted cells [5]. The above described method was applicated first in the treatment of Non-Hodgkin's lymphoma, then chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL) [28]. The use of other antigens may also leverage the versatility of T-cells. More clinical trials have been undergoing: targeting B-cell maturation antigen (BCMA) [27], CD20 [8, 42], CD30 [38], CD33 [21], CD123 [33] and CD138 [12, 15].

Up to date, two CAR T-cell therapies were approved for the treatment of hematologic disorders. The first therapy – "*Tisagenlecleucel-T*" (*Kymriah*™, Novartis Pharmaceuticals Corporation) was accepted by *U.S. Food and Drug Administration* (FDA) on May 2018 and by *European Medicines Agency* (EMA) on August 2018 [47]. This therapy uses anti-CD19 CAR technology for the treatment of:

- patients up to age 25 with B-cell precursor ALL, which is refractory (not responding to previous treatment) or is in second or later relapse.
- adults with relapsed of refractory (R/R) DLBCL after two or more lines of systemic therapy [23].

Kymriah™ contains reprogrammed CAR T-cells identifying CD19 through extracellular scFv fragment from monoclonal antibody connected with intracellular signaling domains: 4-1BB (CD137) and CD3ζ [9].

The second CAR T-cell therapy registered so far by FDA and EMA is "*axicabtagene ciloleucel*" (Yescarta™, Kite Pharma Incorporated). It is indicated for the treatment of adult patients with R/R large B-cell lymphoma after two or more systemic therapy, including DLBCL not otherwise specified, primary mediastinal large B-cell lymphoma (PMBCL) and DLBCL arising from follicular lymphoma. *Axicabtagene ciloleucel,* by analogy to *Tisagenlecleucel-T,* targets CD19 as well, however CARs are built of extracellular scFv domain and co-stimulatory CD28 and CD3ζ molecules [9].

The percentage of complete remissions, while using anti-CD19 CAR T-cells, ranges from 70% to 94% in different trials [28]. Available therapies are designated only for autologous application and cannot be used for other patients as allogenic T-cells [50]. About 2 to 14 days before CAR T-cell infusion, recipient should be prepared in advance, by receiving low-dose lymphodepleting chemotherapy. Lymphodepleting regimens contains fludarabine (i.v. four days daily) and cyclophosphamide (daily for two days starting with first dose of fludarabine). Lymphodepletion (destruction of host's lymphocytes prior to immunotherapy) boost the potential of CAR T-cells through increased cytokine release, elongation of their persistence, promoting the proliferation and, in effect, enhance CAR T-cells expansion [4].

Antigen CD19 is typically expressed by all B-cells (healthy and malignant) and does not appear on solid body tissues – neither neoplastic nor healthy [44]. It makes CD19 molecule an selective antigen for the treatment of cancers derived from B-cells. Moreover, hematological cancer cells are void of protective tumor microenvironment (TME) characteristic of solid malignancies and reside in routine locations of T-cell migration (peripheral blood, lymph nodes, bone marrow), which enables CAR T-cells an direct interaction with targeted cells [11]. The above-mentioned factors make the CAR T-cell therapy an efficient option for hematological patients.

Despite the advantages related to anti-CD19 CAR T-cell therapy, the treatment may lead to B-cell aplasia [36]. The effect is an result of CD19 expression on normal B-cells. This adverse reaction may require antibiotics or γ-globulin administration in order to protect patient against serious infections. During these studies also other specific side toxicities were observed – including neurotoxicity and cytokine release syndrome (CRS) [6].

## CAR T-CELLS TARGETED ON SOLID TUMORS

### The challenges facing solid tumors

Since CAR T-cells therapy demonstrated promising outcomes by targeting CD19 towards blood malignancies, there has been a great interest in expanding the CAR technology to the treatment of solid cancers. However, targeting solid tu-

mors is more challenging than struggling against blood cancers. There are several obstacles which prevent the efficacy of CAR-T cells in solid tumors to reproduce the success of hematological counterparts:

1. *Genetic instability of tumor cells*. Tumor cells can stop the expression of surface antigens targeting by CAR T-cells or change their structure. This antigen escape concerns both leukemias and solid tumors. However the heterogeneity of solid tissues predisposes more to deficiency of specific antigens [2, 24].
2. *Tumor immunosuppressive microenvironment*. The histologic properties of solid tumors determine difficult conditions for engineered T-cells. The dense stroma of a tissue serves as a physical barrier for direct penetration of CAR T-cells to cancer cells. Components of stroma may be a source of inhibitory cytokines, such as IL-6, TGF-β or IL-10 [37]. Moreover, TME is characterized by chemical properties reducing therapy effectiveness, including hypoxia and low pH. Together these factors can restrict the effector functions of CAR T-cells, due to loss of ability to traffic the tumor locus [3].
3. *Lack of targetable antigens*. Another challenge in developing CAR T-cells includes lack of cell surface tumor-specific molecules in various types of cancers. The ideal target epitope should be expressed on every cancer cell. However, some tumors, like pancreatic cancer do not present abundant antigenicity. Moreover, some targeted antigens are expressed on non-malignant healthy tissues as well (usually in a smaller quantity). It may be the reason of undesirable, cross-reaction of CAR T-cells and therapy side effects [1, 29].

## Present achievements in solid tumors treatment

Despite above-mentioned barriers, a number of recent studies demonstrate the effectiveness of CAR T-cell therapy against solid tumors. At present, over 450 CAR T-cell trails are registered in the U.S. National Library of Medicine (*clinicaltrials.gov*). About one third of them investigate the usefulness of CAR T-cell therapy against solid tumors [34]. However, none of them are accepted for conventional therapy. Currently taken approaches include CAR T-cells targeted against various cancer surface epitopes: epidermal growth factor (EGFR) in the treatment of non-small cell lung cancer, the carcinoembryogenic antigen (CEA) against adenocarcinoma liver metastases, disialoganglioside 2 (GD2) for neuroblastoma, the human epidermal growth factor receptor 2 (HER2) against sarcoma and gliomas, ephrin type-A receptor 2 (EphA2) for gliomas and non-small cell lung cancer, CD133 and mucin 1 (MUC1) for liver carcinoma and pancreatic ductal adenocarcinoma, mesothelin (MSLN) for mesothelioma and ovarian cancer, the prostate-specific membrane antigen (PSMA) for prostate cancer and interleukin-13Ra2 (IL-13Ra2) against glioblastoma and others: L1 cell adhesion molecule (L1-CAM), CA125, fibroblast activation protein (FAP), cancer/testis antigen 1B (CTAG1B), Glypican-3 [52, 53].

Number of other studies is constantly carried out to improve therapeutic efficacy of CAR T-cells against solid tumors. Some of them are directed to target tumor microenvironment, e.g. using nano-based CAR T-cells with EIIIB$^{+}$ fibronectin variants against tumor vasculature and stoma [46]. Moreover, attempts are made to combine chimeric receptors into more complicated structures able to bind more antigens – bispecific CAR T-cell therapy [19]. Methods combining CAR T-cell therapy with supportive cytokines administration are also taken into consideration to enhance T-cells activity [35].

## CONCLUSIONS

CAR T-cell therapy presents an countless potential to revolutionize immunotherapy of cancer. The treatment with CAR T-cells is an antigen-specific method matched for the particular patient's disease. In recent years the huge progress has been made to prove the effectiveness of CAR T-cells therapy. The current and the available knowledge on CAR T-cells is only the beginning of the exploring future potential of this therapy.

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