

Implementation of Small Molecule Drugs and Advanced Techniques in CAR-T Cell Therapy

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Abstract. Cancer immunotherapy has developed at a high speed in recent years, and CAR-T therapy is now one of its representative forms. However, the clinical application of CAR-T therapy is also severely restricted by the immunosuppressive tumour microenvironment and is not yet widely available. The following are a few characteristic features of CAR-T cells, the structure and metabolism of the tumor microenvironment, the function of small-molecule drugs, and the progress of new advanced technologies in this field. Research on the regulatory impact of small-molecule drugs in the tumour microenvironment and its application value in economics, safety and efficacy. This paper will first introduce the development trajectory and existing deficiencies of CAR-T therapy, list the merits and defects of small-molecule drugs based on their drug-binding mechanisms, and then discuss how new support tools can alleviate the problems and risks of small-molecule interventions. There are also current deficiencies in the research, and some suggestions for the next round of exploration are offered. Systematically collect professional contents and provide a unified interpretation of special terms.

Keywords: CAR-T, tumor microenvironment, small molecule drugs

1. Introduction

The Development of Cancer Treatment in Biomedicine Continues to Progress. With the development of industry and modern life, changes in the environment have increased people's risk of developing malignant tumours, and as a result, the number of cancer cases around the world has risen. Based on the clinical problem, many new and excellent anti-tumor treatments have been developed continuously, and CAR-T therapy is one of the outstanding achievements. CAR-T therapy fuses T cells with synthetic chimeric antigen receptors (CAR). Many clinical trials have shown good results for various types of leukaemia and B-cell lymphoma in the targeted high-efficacy settings for CAR-T cell therapy. CAR-T therapy has shown a relatively low therapeutic effect in solid tumours due to an adverse tumour microenvironment (TME).

Solid tumours have low TME, are structurally dense and metabolically inactive. Immunosuppressive cell populations in this process include regulatory T cells (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs), all of which suppress the infiltration and activity of effector immune cells. In addition, other reasons for the impaired transport and invasion of immune cells include an excess of extracellular matrix and

fibrosis, as well as abnormal tumor blood vessels; thus, the therapeutic effect of immune cell-based therapy may be diminished. In addition, metabolic factors such as hypoxia, nutrient deprivation and the build-up of immunosuppressive metabolites, for example, adenosine and lactate, will harm the survival and function of T cells in the TME [1]. An abnormally high osmotic pressure in the space between cells can prevent the CAR-T cells from exiting the tumour and thus harm the tumour cells. Small-molecule drugs have been developed to modify a hostile TME and promote the infiltration of CAR-T cells into the tumour site.

At present, the main directions of regulatory strategies are to promote the expression of tumour antigens and to normalise the tumour vasculature. Decitabine and EZH2 inhibitors promote the apoptosis of tumour cells by removing or inhibiting methylation modification at tumour suppressor genes. Lenvatinib and Apatinib reduce the number of growth factor receptors on the surface of tumour cells. Therefore, it does not bind to the receptor kinase, prevents the activation of the proangiogenic signal pathway, and inhibits receptor autophosphorylation. Although the above interventions have been shown to have some therapeutic effects, there are also risks, such as inflammatory storm, general toxicity from drugs, off-target damage and vascular occlusion. The above are not severe risks to patient safety. At the present time, high-end technologies such as Boolean Logic CAR, Suicide Gene systems, vascular perfusion techniques and multiphoton microscopy can be applied to address adverse reactions and enhance the all-around effectiveness of CAR-T cells. Based on the above research results and clinical data, this paper reviews the regulatory path for small-molecule drugs that enhance conventional CAR-T therapy and offers theoretical support for the future development of cancer combination treatment strategies.

2. CAR-T therapy

CAR is a module synthetic receptor that has the following four components: an extracellular target antigen-binding domain, a hinge region, a transmembrane domain, and one or more intracellular signalling domains [2]. The antigen-binding domain is a fusion of the variable heavy and light chains, referred to as a single-chain variable fragment (scFv), that specifically binds to antigens on the surface of tumour cells. The Hinge Region provides a degree of freedom to reduce steric hindrance and allows the antigen-binding domain to access the target epitope. The transmembrane region of CAR binds to the T-cell membrane and promotes the expression and stability of CAR. Phosphorylation of the intracellular signalling domain occurs after receiving an activation signal, recruits downstream signalling molecules, and thus triggers T-cell activation.

CAR-T cells have been continuously structurally optimised to improve therapeutic efficacy. Over the past few years, changes have occurred in the composition of the intracellular regions, and as a result, CAR has also been modified. The first-generation CARs only had a single intracellular CD3 zeta-derived signalling domain. Although these CARs could activate T cells, they showed poor anti-tumour activity in vitro. As a result, second-generation CARs were developed to add an intracellular costimulatory domain in addition to the CD3 zeta-signaling domain. To further enhance the anti-tumor effect of CAR-T cells in the future, a third generation of CARs has been developed, and among them are two intracellular cis-acting co-stimulatory domains in addition to CD3 zeta. Afterward, the fourth-generation CARs were modified at their intracellular domains to enable the expression of cytokines in a tenting or structural manner based on the derived second-generation CARs, by modifying their intracellular domains for inducible or constitutive expression of cytokines [2].

3. The tumor microenvironment

3.1. Immune suppression

The features of a tumour microenvironment (TME) are generally immunosuppressive, have a dense physical structure, and are metabolically hostile.

Solid tumours are proliferation environments supported by immunosuppressive cell types, such as Tregs, TAMs switched to an anti-inflammatory M2 phenotype, myeloid-derived suppressor cells (MDSCs) that originate from myeloid cells, and cancer-associated fibroblasts (CAFs) linked to cancer. These cells release various immunosuppressive factors, such as transforming growth factor-beta (TGF- β), interleukin-10 (IL-10) and vascular endothelial growth factor (VEGF) [3]. These mediators reduce the development of dendritic cells (DCs). DCs are required for antigen presentation and T-cell activation to trigger a cytotoxic anti-tumor immune response. Reduced mature DC populations limit the proliferation and activation of cytotoxic T lymphocytes (CTLs), thereby inhibiting the induction of a systemic anti-tumor immune response. Prolonged tumour growth is beyond the tumour clearance capacity of the weakened immune system; thus, the disease will continue to spread and the patient's general health will decline.

3.2. Dense structure

The main reasons for the dense physical structure of solid tumors are the abnormal deposits of extracellular matrix (ECM). An extensive extracellular matrix (ECM) has developed, which is composed of dense collagen fiber networks with a high level of hyaluronan, and this structure physically obstructs the infiltration of immune cells and restricts the spread of therapeutic drugs to the tumor core. Tumor fibrosis is likely to worsen the problem further by increasing the rigidity of the extracellular matrix (ECM) and reducing the functional perfusion of intratumoral vessels; this is mainly driven by activated cancer-associated fibroblasts (CAFs) [4]. Normal ECM maintains interstitial connections and tissue stability; tumour cells increase cross-linking of ECM and fill the interstitial space, forming a stiff, thick, sticky stromal network. Tumor fibrosis also damages the microenvironment by altering the structure of blood vessels irregularly; thus, the shape of these vessels is deformed, and blood supply is reduced due to vascular leakage. The above reasons are due to reduced blood supply and a high internal pressure. Drugs and immune cells are transported in the blood in vivo, and abnormal vascular structures create ischaemic areas that are difficult to reach by drugs. Vascular leakage and ECM compression further increase intratumoral pressure; thus, the drug and immune cells cannot enter the tumour to take effect.

3.3. Hostile metabolism

Hypoxia induces tumour adaptation by activating hypoxia-inducible factors (HIFs), promoting enhanced anaerobic glycolysis (Warburg effect), overproduction of lactate, and tumour extracellular acidification. Lactate, adenosine and kynurenine are produced in the tumour microenvironment; together they suppress the cytotoxic activity and division of T lymphocytes and other immune cells [5]. Tumor blood vessel growth cannot meet the demand for oxygen by rapid tumour enlargement. Abnormal vascular structure and high metabolic demand of the tumor cells also exacerbate hypoxia. Metabolites inhibit the activity of cytotoxic T lymphocytes through several mechanisms, such as interfering with energy metabolism, blocking NFAT signalling, regulating extracellular CD44 receptors, inhibiting PKA-mediated mTORC1 activation, inducing T-cell exhaustion, suppressing

dendritic cell (DC) antigen presentation, depleting tryptophan, and inhibiting amino acid uptake. Metabolic reprogramming at the same time promotes the polarisation of M2 macrophages, increases the functional activity of Tregs and MDSCs, and induces the differentiation of naive T cells into Tregs for immunosuppression. The above adverse features of TME jointly restrict both the clinical application and efficacy of CAR-T therapy in multiple ways.

4. Small molecule drugs

4.1. Decitabine and EZH2 inhibitors

Decitabine and EZH2 inhibitors are both small-molecule drugs that target antigens. Decitabine is a demethylating agent that reawakens silenced tumour suppressor genes by reversing abnormal DNA methylation. The main path is as follows: It is first converted into the triphosphate form in the body and, acting as a mimic of cytosine, joins DNA synthesis. DNA methyltransferase (DNMT) initiates the methylation reaction by binding tightly to simulated cytosine analogues, thus resulting in covalent trapping and functional inhibition of DNMT.

EZH2 is a histone methyltransferase that regulates the transcription of tumor suppressor genes through histone modification. EZH2 inhibitors inhibit the epigenetic modification mechanism of this way. Most EZH2 inhibitors function by competing with S-adenosylmethionine (SAM) for the active binding site of EZH2. SAM depletion inhibits EZH2-mediated methylation of histone H3 lysine 27 (H3K27me3), blocks epigenetic histone modifications, and thus reactivates silenced tumor suppressor genes.

4.2. Lenvatinib and Apatinib

Lenvatinib and Apatinib are both small-molecule drugs that inhibit tumor angiogenesis. Lenvatinib is a multi-target receptor tyrosine kinase inhibitor. The above mainly operate on VEGFR1-3, FGFR1-4, PDGFR α , KIT and RET. Selectively inhibit the kinase activity of the above receptors to block proangiogenic signalling pathways.

Apatinib is a type of small-molecule anti-angiogenic drug. It is a VEGFR-2 agonist. It can bind to the ATP-binding site inside the cell and prevent autophosphorylation of VEGFR-2.

4.3. Practicality

The three essential aspects of practical application value are economically viable, clinically safe and therapeutically effective; only when all three are met can these small-molecule drugs be promoted in clinical trials and applied to patients. Small molecule targeted drugs are of different types and thus have various differences in administration routes, medical insurance coverage, economic costs and pharmacoeconomic benefits; accordingly, their practical values in real-life clinical practice also vary significantly. Collectively, the above indicators can provide objective data to select suitable clinical drugs and make personalised treatment choices.

Each of these drugs has different strengths and weaknesses in terms of clinical application costs and patient affordability. Decitabine's current administration is generally by intravenous infusion, which requires more personnel, time and other resources. Fortunately, an oral-administration method for Decitabine has been developed recently and is no longer required. Analysis shows that the total non-drug administration cost per patient is €3574.6 and €781.4 for a treatment cycle of IV and oral decitabine, respectively; thus, the cost impact of oral drug introduction is -€2793.2 (-78.1%) [6].

Although medical insurance reimbursement reduces the financial pressure on the public, its application is cumbersome and it has not spread widely. EZH2 inhibitors are taken orally and have good patient adherence; however, as new targeted drugs, they are relatively expensive and not yet covered by most medical insurance schemes abroad. On the other hand, Lenvatinib is orally available and widely covered by medical insurance in many parts of the world. Apatinib is also taken orally for convenience, and studies on the cost-effectiveness of targeted therapies in the Chinese population have found that apatinib is more cost-effective than other targeted drugs, such as lenvatinib. According to the above data, apatinib therapy has been shown to achieve a 0.837 increase in quality-adjusted life years (QALY) and a cost saving of US\$6,975 compared with lenvatinib. Sensitivity analysis has further shown that the cost-effective advantage of apatinib is stable and independent of patients' willingness-to-pay in China [7], and subgroup analysis also confirms that apatinib can maintain a longer progression-free survival (PFS) at a lower economic cost, thus having outstanding economic advantages for clinical use.

These small-molecule drugs have all demonstrated some therapeutic effects and side-effect profiles in terms of clinical safety and efficacy. Decitabine and EZH2 inhibitors have shown similar clinical efficacy to conventional treatment regimens, but their survival benefits for particular subgroups of patients are relatively small and are accompanied by severe blood disorders. The first side effect of decitabine is reduced blood cell count (myelosuppression), including neutropenia, thrombocytopenia and anaemia; these are expected given its nature as a drug. A retrospective clinical study of Chinese patients with intermediate- to high-risk myelodysplastic syndrome (MDS) showed that the complete response rate (CR) of decitabine treatment was 44.9% and the overall response rate (ORR) was 67.3%. Progression-free survival reached 9 months in the single-agent group and 13 months in the combination chemotherapy group, with no significant difference between the two groups [8]. EZH2 inhibitors have well-known and predictable hematological adverse effects, such as thrombocytopenia, neutropenia and anaemia, and the primary possible clinical risk is secondary malignancy. EZH2 inhibitors have good safety and strong anti-cancer effects in treating epithelioid sarcoma and non-Hodgkin's lymphoma of resistant solid tumours. Lenvatinib has been shown to prolong the progression-free survival and overall survival of tumor patients effectively; based on systematic reviews, it has achieved superior improvements in PFS and OS for HCC patients compared with sorafenib, but it is associated with a relatively high frequency of side effects, including reduced appetite, proteinuria, dysphonia, weight loss and hypothyroidism [9]. Apatinib also has a high overall incidence of adverse reactions, but most of the toxic reactions, such as hypertension and hand-foot syndrome, are clinically manageable. A series of previous meta-analyses have verified that apatinib can effectively treat relapsed and refractory osteosarcoma with good safety in [10]. Apatinib has a relatively small cost of treatment, is therefore more feasible for application in certain groups and under specific medical systems.

5. Modern methods

5.1. Boolean Logic CAR

The basic idea of Boolean Logic CAR is to make engineered T cells accurately recognise antigens and then selectively kill tumour cells by combining signals from multiple antigens through Boolean logic operations. The three basic gate modes are AND, OR, and NOT. Only under certain antigen combination conditions will T cells be activated to distinguish between cancer cells and healthy cells more accurately.

Neither decitabine nor EZH2 inhibitors have eliminated intratumoral antigen heterogeneity and antigen escape entirely. Tumor cells have evaded the immune system by several ways, such as mutations in antigen genes, defects in antigen processing, lineage switching, membrane redistribution, cytoskeleton-mediated epitope masking, and masking during CAR-T cell manufacturing. Boolean Logic CAR blocks tumor immune escape induced by single antigen variation through logical gating design to address the problem of tumor heterogeneity caused by small-molecule drugs.

Boolean Logic CAR-T cells have been shown *in vivo* to maintain the ability to recognize targets and kill tumour cells without repeated periodic administration, thus providing a possible alternative to long-term oral multi-target inhibitors such as lenvatinib and apatinib. It can be more precisely directed to reduce off-target toxicity caused by the systemic administration of small-molecule anti-cancer drugs [2, 5].

The purpose of a suicide gene system in CAR-T cells is to act as a safety mechanism; that is, in the event of excessive immune activation or severe side effects, such as cytokine release syndrome (CRS) and neurotoxicity, these cells will be quickly eliminated within a short period. The two most mature suicide gene systems are iCaspase-9 and HSV-TK; specifically, iCaspase-9 is constructed by fusing human caspase-9 with modified FK506-binding protein (FKBP12), and the addition of the small-molecule inducer AP1903 induces FKBP domain dimerization to activate the downstream caspase-9 pathway and rapidly cause apoptosis in CAR-T cells; the HSV-TK system uses herpes simplex virus-derived thymidine kinase (HSV-TK) gene, and after administration of the prodrug ganciclovir (GCV), HSV-TK phosphorylates GCV into active metabolites that incorporate into cellular DNA, inhibit DNA synthesis, and induce cell death in CAR-T cells. The main adverse reactions of CAR-T therapy include cytokine release syndrome (CRS) and related neurotoxicity, and in large-scale clinical application of EZH2 inhibitors such as talazoparib and SHR2554, the incidence of grade 3 and above hematological toxicities is approximately 33%, with a treatment-related mortality of about 2%. Therefore, a suicide gene system is used as an active safety mechanism to eliminate excessively activated immune cells in the event of severe adverse effects during combination therapy and improve biosafety.

5.2. Vascular Perfusion Techniques

Vascular Perfusion Techniques are interventional therapeutic strategies for localised delivery of high-concentration drugs to tumour sites while reducing systemic toxicity, and the three main development directions are as follows: TAMP is a technology that uses an adjustable double-balloon occlusion catheter to create a region of reduced blood flow at the tumour feeding artery by inflation, then introduces high-pressure drug infusion through the newly opened window to promote the passive transport of small-molecule drugs to nearby tumour cells. Surgical and minimally invasive routes to achieve target-organ vascular isolation can be used to locally administer high-dose therapeutic drugs at the site of disease and reduce their systemic absorption by lowering blood concentration. Optimize the hemodynamics and perfusion pressure within the tumour to enhance the delivery efficiency of systemic drugs and immune effector cells, and promote the aggregation of small-molecule inhibitors and immune cells in tumour lesions. Lenvatinib and apatinib are oral drugs that are readily absorbed by the body and have off-target toxicities, such as hypertension, proteinuria and hand-foot skin reactions; TAMP, on the other hand, uses local arterial infusion of targeted drugs to concentrate high-dose medications at the tumor site, reduces the systemic exposure of therapeutic agents to normal organs, and effectively mitigates the dose-limiting toxicity of targeted small-molecule drugs. Combination therapy of decitabine, EZH2 inhibitors and anti-

angiogenic drugs, such as lenvatinib, also has some deficiencies; specifically, these drugs are distributed unevenly in the tumour, and tissue permeability is poor. For hypovascular tumours in particular, traditional arterial chemotherapy shows reduced efficacy, and TAMP can enhance the infiltration and diffusion of small-molecule drugs throughout the entire tumour by increasing high-pressure perfusion and regulating the microvasculature; thus, it improves drug exposure in hypoperfused areas and further enhances the therapeutic effect of small-molecule intervention strategies.

5.3. Multiphoton microscopy

Multiphoton microscopy is a very high-resolution in vivo optical imaging technology that uses secondary or tertiary nonlinear excitation of near-infrared femtosecond lasers. Reduce phototoxicity and photobleaching; excite fluorescence signals only at the focal plane; support long-term, deep and three-dimensional dynamic imaging of living tissue microenvironments.

Four applications of multiphoton microscopy in the study of tumor immunotherapy are: real-time dynamic tracking of lymphocytes, imaging of vascular permeability and depth of perfusion, non-invasive observation of metabolic and immune microenvironment changes, and live-cell observation of intracranial drug delivery.

After treating with the anti-angiogenic TKIs lenvatinib and apatinib, damaged tumour microvessels are likely to leak irregularly. Dynamic fluorescence imaging and two-photon microscopy under multiphoton microscopy can be used to observe the effect of drugs on tumour blood supply and distribution efficiency in real time, and accurately evaluate changes in vascular function; thus, indirect measures based solely on the size of the tumour are avoided. Provide direct experimental support for identifying the optimal time window of vascularisation normalisation in combination therapy.

6. Conclusion

The functional mechanism of the main fourth-generation CAR-T is a whole cascade: scFv recognizes tumor-specific antigens without the need for MHC, and the intracellular signalling domain activates T cells to form an immune synapse with tumour cells. Activated CAR-T cells release perforin and granzyme to kill tumour cells, and costimulatory domains can be employed to induce the differentiation of memory T cells for prolonged anti-tumour immunity. Immune suppression in the tumour microenvironment (TME) is due to the impaired maturation of dendritic cells (DCs), dense physical barriers from ECM compression and vascular abnormalities, as well as a metabolically unfavourable niche with nutrient deprivation and accumulation of immunosuppressive metabolites.

Decitabine and EZH2 inhibitors suppress tumour antigenicity; lenvatinib and apatinib regularise tumour vasculature. The former have disadvantages in administration accessibility, cost and haematological toxicity, with limited survival benefits; anti-angiogenic agents are more economically feasible and clinically effective. Boolean logic CAR-T can address antigen escape and off-target toxicity; suicide gene systems can reduce CRS, neurotoxicity and other haematological adverse events to improve the safety of CAR-T. TAMP reduces the delivery of drugs to tumours and therefore lowers systemic toxicity; at the same time, multiphoton microscopy is used to observe changes in cells at a finer level.

Three research directions are thus proposed. First, decitabine/EZH2 inhibitors increase the expression of tumor antigens to induce killing by AND-gated CAR-T cells, and an iCaspase9 suicide

gene is used as a safety switch. Second, TAMP will deliver anti-angiogenic drugs and CAR-T cells sequentially through tumor-feeding arteries to normalise the vasculature and improve immune cell infiltration dynamically, as observed by multiphoton microscopy. Third, an ex vivo and in vivo integrated multiphoton imaging platform will be established using patient-derived tumor tissues to screen optimal medication regimens, and combined with NOT-gate CAR-T for sparing normal tissues.

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