Potential strategies against resistance to CAR T-cell therapy in haematological malignancies

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Abstract: Chimeric antigen receptor (CAR) T-cell therapy is a rapidly developing method for adoptive immunotherapy of tumours in recent years. CAR T-cell therapies have demonstrated unprecedented efficacy in the treatment of patients with haematological malignancies. A 90% complete response (CR) rate has been reported in patients with advanced relapse or refractory acute lymphoblastic leukaemia, while >50% CR rates have been reported in cases of chronic lymphocytic leukaemia and partial B-cell lymphoma. Despite the high CR rates, a subset of the patients with complete remission still relapse. The mechanism of development of resistance is not clearly understood. Some patients have been reported to demonstrate antigen-positive relapse, whereas others show antigen-negative relapses. Patients who relapse following CAR T-cell therapy, have very poor prognosis and novel approaches to overcome resistance are required urgently. Herein, we have reviewed current literature and research that have investigated the strategies to overcome resistance to CAR T-cell therapy.

Keywords: CAR T-cell therapy, drug resistance, haematological malignancies

Introduction

Chimeric antigen receptors (CARs) are synthetic tumour-specific receptors that are genetically reprogrammed in vitro using a patient’s own T lymphocytes, which bind a tumour antigen in a major histocompatibility complex-independent manner, allowing T cells to recognise and kill antigen-expressing cancer cells. In the past few years, clinical trials using CAR T cells have demonstrated high rates of response in the treatment of patients with haematological malignancies, as well as increased duration of remission in patients with acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), and partial B cell lymphomas. CAR T-cell therapy has provided a new therapeutic option to patients with relapse/refractory haematological malignancies. Based on the results, the United States Food and Drug Administration (FDA) approved tisagenlecleucel in August 2017 for paediatric patients and young adults with B-cell ALL (B-ALL). Furthermore, in October 2017, the FDA approved CAR T-cell therapy for the treatment of B-cell lymphoma. A current challenge in CAR T-cell therapy is that a portion of the patients achieving remission following CAR T-cell therapy subsequently undergo relapse. The mechanism of development of resistance to CAR T-cell therapy is not completely understood. Some patients have been reported to demonstrate antigen-positive relapse due primarily to shorter duration of persistence of CAR T cells, whereas others show antigen-negative relapses associated with lineage switching, acquired mutation and alternative splicing, epitope-masking and antigen downregulation. The current review outlines the diverse strategies to overcome or reduce resistance to CAR T-cell therapy.

Basic structure and development of CAR T-cells

CAR T-cell therapy is a cellular therapy that redirects a patient’s T cells to specifically target and destroy tumour cells. CARs are proteins expressed on the surface of T and natural killer (NK) cells, thereby enabling them to target and destroy tumour cells. CARs consist of two main components: the extracellular domain, which typically contains a tumour-specific antigen recognition motif, and the intracellular signalling domain, which mediates T-cell activation. The development of CAR T-cell therapy has involved the modification of T cells to express CARs, which are designed to target specific tumour antigens. This allows for the selective targeting of tumour cells, enabling T cells to recognise and destroy them. The success of CAR T-cell therapy has been attributed to its ability to overcome immune evasion mechanisms and to achieve durable clinical responses in patients with haematological malignancies.

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which contain extracellular binding domains, a hinge region that mediates the linkage of extracellular to transmembrane domains, a transmembrane domain and an intracellular signaling domain (Figure 1).16–20 In 1987, Kuwana et al. first proposed the concept of CAR and constructed a prototype of CAR-T cells that specifically recognised tumour-associated antigens.21 In the first-generation CARs, the intracellular signaling domain comprised solely a CD3ζ chain, a component of the endogenous T-cell receptor (TCR).22 These first-generation CARs showed minimal killing and persistence in vivo along with limited clinical benefits.23–28 Second-generation CARs incorporated co-stimulation into the CD3ζ construct. Most investigators work with second-generation CARs, involving those that express the classical co-stimulatory molecules, namely the tumour necrosis factor (TNF) superfamly members 9 (4-1BB) and 4 (OX40).29,30 However, some investigators have expanded their toolkit to include other types of co-stimulatory molecules into the CAR constructs, such as OX40, 4-1BBL, or inducible co-stimulator (ICOS).31–33 Studies have reported that second-generation CAR T cells demonstrated potent expansion and cytokine secretion abilities, and persistence of anti-tumour T cells both in vitro and in vivo.7,17,34–36 Third-generation CARs containing three or more co-stimulatory domains to boost T-cell activation signals, including CD28, 4-1BB, and CD3ζ, were developed to improve the design and enhance the activation of the second-generation CARs.37–45 The fourth-generation CARs (T cells redirected for universal cytokine killing, TRUCKs) can secrete pro-inflammatory cytokines such as IL-12 into the tumour microenvironment,46,47 which consequently improve the tumour eradication ability of these cells.48–52

**Efficacy of CAR T cells in the treatment of haematological malignancies**

Haematological malignancies are one of the most common cancers among patients in China. Presently, haematological malignancies remain incurable and have a high recurrence rate and mortality. In recent years, novel gene and targeted therapies have emerged for the treatment of patients with haematological malignancies;
however, clinical remission rates are limited. In 2013, the journal *Science* summarised the top 10 breakthrough technologies in the scientific community, with tumour immunotherapy topping the list. CAR T-cell therapy, as a special tumour immunotherapy, has demonstrated remarkable results in the treatment of patients with malignant tumours, especially lymphatic haematopoietic malignancies.

**B-ALL**

CAR T-cell therapy has emerged as a highly effective therapy for patients with relapsed or refractory B-ALL with previously limited treatment options. The therapy was reported to demonstrate complete responses (CRs) ranging from 60% to 90% (Table 1).\(^2,7,48-53\) Relapse rates of approximately 30–50% were reported in patients with B-ALL, with the majority being CD19-negative relapses.\(^7\) In a phase II, single-cohort, 25-centre global study, 75 patients received an infusion of tisagenlecleucel and were followed up for at least 3 months; the overall remission rate was 81%.\(^54\) A total of 45 patients (60%) had a complete remission between 6 weeks and 8.5 months after infusion of CAR T cells.\(^2,7,48-53\) Relapse rates of approximately 30–50% were reported in patients with B-ALL, with the majority being CD19-negative relapses.\(^7\)

**Table 1. Summary of CAR T cells in the treatment of B-ALL, B-NHL and CLL.**

| Disease | Patient populations | Response and relapse | References |
|---------|---------------------|----------------------|------------|
| B-ALL   | 53 adults           | 44/53 (83%) achieved a CR, the median overall survival was 12.9 months. | Park *et al.*\(^2\) |
|         | 30 paediatric and adults | 27/30 (90%) achieved a CR, seven patients who had a complete remission subsequently had a relapse between 6 weeks and 8.5 months after infusion of CAR T cells. | Maude *et al.*\(^7\) |
|         | 75 paediatric and adults | 45/75 (60%) had a CR, the rate of overall survival was 90% at 6 months after infusion and 76% at 12 months after infusion. | Kochenderfer *et al.*\(^54\) |
|         | 21 paediatric and adults | 14/21 (66.7%) achieved a CR. | Lee *et al.*\(^53\) |
| B-NHL   | 28 adults           | 6/14 DLBCL patients achieved a CR and 10/14 FL patients achieved a CR. | Schuster *et al.*\(^40\) |
|         | 7 adults            | 4/7 (57%) achieved a CR. Three patients are in ongoing CR at 12 months post CAR T cells infusion. | Locke *et al.*\(^63\) |
|         | 101 adults          | CR rate was 54%. With a median follow-up of 15.4 months, with 40% continuing to have a complete response. | Ye *et al.*\(^66\) |
|         | 15 adults           | 8/15 (53%) had a CR. Seven patients are in ongoing CR, ranging from 9 to 22 months post CAR T cells infusion. | Schuster *et al.*\(^40\); June and Sadelain\(^61\) |
|         | 7 adults            | 2/7 achieved a CR, one patient attained a PR, another four patients exhibited SD. Two patients are in ongoing CR at 3 months and 13 months post CAR T cells infusion. | June and Sadelain\(^61\) |
| CLL     | 14 adults           | 8/14 (58%) achieved an objective response, with 4/14 (29%) achieving a CR. CAR T cells persisted for >5 years in two patients with durable CRs. | Porter *et al.*\(^59\) |
|         | 3 adults            | 2CR, 1PR; two of whom experienced long-lasting CR. | Porter *et al.*\(^59\) |

\(B-ALL, B\)-cell acute lymphoblastic leukaemia; B-NHL, B-cell non-Hodgkin lymphoma; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukaemia; CR, complete remission; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; PR, partial remission; SD, stable disease.
B-cell non-Hodgkin lymphomas and CLL

Previous research has shown remarkable rates of complete and durable remission in patients with CLL56–59 and B-cell non-Hodgkin lymphoma (B-NHL).23,56–61 CAR T-cell therapy has been approved for the treatment of lymphoma in adults, with a lower remission rate of approximately 50–70%.6,62–64 Furthermore, antigen loss has also been observed in such patients.65,66 In a multicentre, phase II trial, 111 patients with histologically confirmed diffuse large B-cell lymphoma, primary mediastinal B-cell lymphoma, or transformed follicular lymphoma were enrolled, of which 101 received Axicabtagene Ciloleucel, an autologous anti-CD19 CAR T-cell therapy. The objective response rate observed in the patients was 82%, and the CR rate was 54%. At a median follow-up of 15.4 months, 42% of the patients continued to demonstrate a response, with 40% showing CR.6 Another clinical trial enrolled 28 adult patients, wherein complete remission occurred in 6 out of 14 patients with diffuse large B-cell lymphoma and in 10 out of 14 patients with follicular lymphoma.59,66 Porter et al. treated 14 adult patients with CLL using CD19 CAR T cells, and reported CR in 4 (29%) patients.59

Underlying mechanisms of resistance

Two main mechanisms have been recognised in relapse following CAR T-cell therapy, including antigen-negative and antigen-positive relapses.

Antigen-positive relapse

Antigen-positive relapse has been assumed to be due primarily to short persistence of CAR T cells; however, it can also occur in association with a suppressive tumour microenvironment.67 The reasons for loss of CAR-T cell persistence are complex and might be difficult to determine in individual patients. According to a trial by Park et al., shorter duration of persistence of the CD19-CD28-ζ CAR T cells employed by Memorial Sloan Kettering Cancer Center (MSKCC) could partially explain the low rate of CD19-positive relapse.6 In another study, although the CR rates in the cohort were robust, early relapse was noted in a subset of patients, associated with loss of

**Figure 2.** Mechanisms of resistance to CAR-T cell therapy. (A) Lentiviral modification of a single leukemic cell allowed for joint CAR19 and CD19 expression on their cell surface, effectively masking the CD19 epitope from CAR T cells. (B) Tumour cells can switch to a genetically related but phenotypically different disease. (C) Tumour cells, through genetic mutations, can either completely lose CD19 receptor expression or modify the CD19 receptor that lack the extracellular epitopes recognised by CAR T cells. (D) Tumour cells downregulate the surface target antigen to levels below those needed for CAR T cells activation.

CAR, chimeric antigen receptor.
CAR T cells in the blood due to an anti-CAR T-cell immune response to the epitopes in the murine single-chain variable fragment (scFv). This mechanism was reported to contribute to the early loss of CAR T cells in some patients after the use of a CAR containing a murine scFv.\(^\text{68}\)

**Antigen-negative relapse**

The reason for antigen-negative relapse was unclear. Since the antigen-negative relapse has been considered a major barrier to CAR-T therapies, studies have uncovered multiple mechanisms responsible for the antigen-negative relapse, which are described below.

**Epitope-masking.** Ruella et al. described a rare case of a 20-year-old man with B-ALL who had suffered three chemotherapeutic relapses. He was enrolled in a phase I trial [ClinicalTrials.gov identifier: NCT01626495] to evaluate the safety, feasibility, and engraftment of CD19-targeted CAR T cells (CTL019) in paediatric and young adult patients with B-ALL.\(^\text{10}\) The patient was in complete remission at day 28 after infusion. However, he experienced relapse after 9 months of the CAR T-cell therapy with CD19-negative disease. Ruella et al. probed this patient’s CD19-negative relapse, ruling out CD19 mutations and splicing variants, and found that the anti-CD19 CAR had been introduced into a single leukaemic B cell during manufacturing of the CD19 CAR T cell. This tumour clone was infused into the patient alongside the CAR T-cell therapy and eventually expanded, which led to disease progression and death. The study reported that resistance to CTL019 occurred not due to loss of target by the leukaemic blasts, but due to the CAR molecule bound to adjacent CD19, which effectively masked the CD19 epitope from CART cells in the patient. Ruella and his team were able to create a model of this phenomenon, known as ‘epitope masking’ (Figure 2A), both in vitro and in vivo.

**Lineage switch.** Lineage switch occurs when a patient experiences relapse with a genetically related but phenotypically different malignancy (Figure 2B), which might be a mechanism for antigen loss after CAR T-cell therapy observed in clinical trials.\(^\text{69–72}\) Evans et al. reported relapse after CD19 CAR T-cell therapy in a patient with CLL with Richter’s transformation. The patient demonstrated a plasmablastic lymphoma, which is inherently CD19-negative.\(^\text{73}\) A group of researchers from Seattle reported that two of the seven patients with B-ALL harbouring rearrangement of the mixed lineage leukaemia gene experienced relapse with CD19-negative AML following treatment with CD19 CAR T cells.\(^\text{9}\)

**Receptor genetic mutations.** Acquired mutations and alternatively spliced CD19 alleles in the malignant B cells are other mechanisms for CD19-negative relapse following CD19-targeted CAR T-cell therapy (Figure 2C). Sotillo et al. found that exon 2 of CD19 was frequently spliced, leading to the disappearance of the CD19 epitope, which is recognised by CART cells.\(^\text{8}\) In addition, they observed the variants Aexon-5, 6, which lack the transmembrane domain of CD19, thereby leading to loss of surface expression. Furthermore, they observed that the CD19 protein was present in some patients; however, it was truncated due to lack of the epitope required to trigger CAR T cell recognition for lysis. The Orlando trial identified mutations in CD19 in all 12 specimens at the time of relapse.\(^\text{74}\) Mutations were found throughout exons 2–5 of CD19. Encoding of the transmembrane domain begins at exon 5; therefore, variants in exons 2–5 have been predicted to lead to a truncated protein lacking membrane anchorage. Each patient in the study had at least one unique frameshift insertion or deletion and, in some cases, missense single nucleotide variants were also observed, and alternative splicing occurred with rare frequency. Therefore, acquired mutations and alternative splicing could be other possible mechanisms responsible for antigen-negative relapse.\(^\text{75,76}\)

**Antigen downregulation.** Partial antigen loss may be considered a mechanism for resistance to CAR T-cell therapy due to antigen downregulation (Figure 2D).\(^\text{11–15}\) During the course of antigen recognition, natural TCRs produce a highly organised immune synapse that can recognise an antigen at a very low density.\(^\text{77,78}\) However, the immune synapse created during antigen recognition by CARs is less organised than that by a natural TCR.\(^\text{79}\) These distinctions are likely to significantly affect the quality of responses induced in T cells expressing CARs. Fry et al. observed that relapses in patients treated with a CD22 CAR were associated with diminished and variable CD22 site density on B-ALL cells.\(^\text{11}\) The investigators further demonstrated in animal studies that differential levels of CD22 on leukaemic cells could have a dramatic impact on the anti-cancer efficacy. Another study using a CD20 CAR demonstrated that a threshold level of around 200
antigen molecules per target cell was required to induce lysis, while approximately 10-fold higher numbers of molecules were needed to stimulate cytokine production. Therefore, the signal strength and effector function of CARs might be limited by density of the tumour antigens.

**Overcoming resistance to CAR T-cell therapy**

**Improving CAR T-cell design**

**Selection of effector T cells.** Effector T cells are the main processing plant for the biological activity of CARs, and play a crucial role in the anti-tumour effect and duration of action of CARs. Accurate detection and isolation of the most potential subpopulations of T cells before in vitro expansion can improve the outcomes of CAR T-cell immunotherapy. The cytotoxic activity and proliferative capacity of effector memory T cells are superior to that of central memory T cells (TCM) in vitro; however, TCMs have the potential to induce immune memory, as well as exert a longer lasting anti-tumour activity. Stem cell memory T cells (TSCM) have the property of persistent self-renewal; therefore, these cells have the potential for high proliferation and persistence. Thus, the process of inducing the conversion of CAR T cells into TCMs and TSCMs could be an alternative method to prevent antigen-positive recurrence by enhancing the response and persistence of the cells.

**Antigen density.** Numerous studies have demonstrated that antigen density on tumour cells correlates with the efficacy and remission durability of CAR T cells in patients with leukaemia and lymphoma. A recent research demonstrated that upregulation of CD22 on the cell surface improved CAR T cell functionality and long-term persistence. Moreover, Bryostatin 1, a drug that is being administered safely to humans, can increase the expression of CD22 in leukaemia and lymphoma cell lines, resulting in longer duration of in vivo response. Another research found that γ-secretase inhibitors could markedly increase surface levels of BCMA on myeloma cells, thereby improving tumour recognition by CAR T cells in vitro and enhancing anti-tumour efficacy of BCMA-targeted CAR T-cell therapy.

**Selection of co-stimulatory molecules.** The co-stimulatory molecules in the intracellular signalling region of the CAR T cells play an important role in regulating T cell expansion, duration, and anti-tumour effects; however, the biological activities of individual costimulatory molecules are different. Common costimulatory molecules include CD28, 4-1BB, OX40, ICOS, and CD27, of which CD28 and 4-1BB can effectively promote the secretion of IL-2 and IFN-γ. Studies have shown that 4-1BB can effectively promote the expansion of memory T cells and reduce the depletion of persistent CAR T signals. Thus, CAR T cells incorporating a 4-1BB costimulatory molecule might lead to a reduced antigen-positive relapse. Hombach et al. proved that CAR T cells containing CD28 costimulatory molecules were more effective than those containing CD28-OX40, because CD28-OX40 are capable of promoting activation-induced cell death and reduce anti-tumour activity. Other studies have shown that CAR T cells with two co-stimulants (such as CD28 and 4-1BB) were more effective in improving the survival and cytotoxicity of T cells than CAR T cells with a single co-stimulatory molecule. The above studies indicate that co-stimulatory molecules greatly affect the efficacy of CAR T-cell therapy; however, further in vitro and in vivo research is necessary to determine the optimal type and number of co-stimulatory molecules.

**Fully human CARs.** Presently, clinical trials commonly use the CAR scFv segment of murine origin, which has high affinity and immunogenicity. CAR T cells with high affinity have poor ability to distinguish tumour cells with high levels of target antigen from normal cells with low expression. Furthermore, the human body will reject CARs with high immunogenicity, considering them foreign bodies. Reducing immunogenicity of CARs using fully human scFvs could improve the persistence of CAR T cells and their functions against tumour cells. Sommermeyer et al. reported that CARs constructed from fully human CD19-specific scFvs exhibited superior function in vitro and in vivo compared with the FMC63 CAR utilised in clinical trials. Specifically, fully human CD19-specific scFvs were more effective in lysing CD19+ target cells, produced higher levels of cytokines, and proliferated more after activation compared with murine scFv.

**Armoured CAR T cells.** Armoured CAR T cells are modified to co-express cytokines and co-stimulatory molecules in order to enhance the anti-tumour immune response by converting a suppressive tumour microenvironment into a proinflammatory one. For example, CAR T cells armoured to
secrete IL-12 enhance the cytotoxic activity of CD8+ T and NK cells and stimulate a Th1 helper T cell response.93 CD40L expressed in armoured CAR T cells increased the cytotoxicity of these cells in vitro and prolonged survival of lymphoma-bearing mice. CART cells armoured with 4-1BBL has been reported to exert immunostimulatory effects.94 However, the effectiveness of this approach needs further clinical translation as the results have been predominantly proven on pre-clinical models.

**Universal CAR T cells.** Universal CAR T cells are used in genome-editing technologies such as zinc finger nuclease, transcription activator-like effector nuclease (TALEN) and CRISPR-Cas9 to knock out TCR, human leukocyte antigen and other related signaling pathway genes on donor T cells,95 thereby reducing the risk of graft-versus-host disease and immune rejection. Furthermore, simultaneously knocking out immune checkpoints such as programmed death-1 (PD-1) has been shown to enhance the function of CAR-T cells.95–98 Waseem et al. used TALEN to knock out TCR and CD52 on CART cells targeting CD19 for the treatment of patients with refractory ALL, and reported improvement in the condition of the patients within 28 days after treatment. The results proved the safety and effectiveness of universal CAR T-cell immunotherapy for the first time.97 Therefore, use of universal CAR T cells might be another possible strategy to overcome resistance to CAR T-cell therapy.

**Multi-targeted CAR T cells.** Strategies to overcome the relapse rate due to antigen loss following CAR T-cell therapy can be combined with the following approaches: (a) T-cell products that are separately transduced for different CARs can be infused together or sequentially; (b) use of a single vector that encodes two or three different CARs on a single T cell (bicistronic CAR); or (c) encode two CARs on the same chimeric protein using a single vector (tandem CAR) (Figure 3). Majzner et al. described these different approaches in a review.12 Many of these approaches are currently being investigated in clinical trials on patients with haematological malignancies.11,99–101 A recent study investigated the clinical efficacy of bispecific tandem CAR T cells directed against both CD19 and CD20 antigens in patients with relapsed/refractory (R/R) B-cell NHL.102 In addition, Ruella et al. reported that dual CAR CD19 and CD123 overcame both antigen escape and lineage switch.103 Several clinical trials are underway to test multi-specific CAR T cells; however, the effectiveness of these approaches remains to be established.

**Improvement of tumour immune microenvironment**

Improving the tumour immune microenvironment can greatly improve the immune efficacy of CAR T cells and reduce the adverse events. However, due to the complexity of the tumour microenvironment and the diversity of regulatory mechanisms, clinical efficacy cannot be achieved by monotherapy. The main regulatory mechanisms of the immune microenvironment can be combined with the following comprehensive treatments.

Studies have proved that addition of an agent blocking the PD-1 immunosuppressive pathway
(anti-PD-1) greatly improved the efficacy of CAR T cells by inhibition of the interaction between PD-1 and its ligands PD-L1/PD-L2.104,105

Similarly, chemotherapy and radiotherapy can improve immunosuppression by inducing apoptosis of or specifically removing regulatory T cells (Tregs). Moreover, eradication of Tregs can enhance the cell response and increase levels of CAR T cells.106 One study found that chemotherapy based on low-dose cyclophosphamide could effectively eliminate Tregs and exert immunomodulatory effects. A combined immunotherapeutic approach has been reported to improve the prognosis.107

The cytokines TGF-β and IL-10 are the major immunosuppressive factors, and downregulate the expression of TGF-β and IL-10 receptors on T cells by genetic engineering methods, to improve the efficacy of CAR T cells. In addition, activation factors such as IL-2, IL-12, and IL-15 can promote the immune function of effector T cells by creating a microenvironment that is conducive to the survival and efficacy of T cells, resulting in more effective anti-tumour effects by inducing the secretion of activating factors by CAR T cells.47,108,109

Combining CAR T-cell therapy with other agents, such as Bruton tyrosine kinase inhibitors, may reduce recurrence after infusion and improve long-term survival. Fraietta et al. reported that treatment with ibrutinib could significantly increase the implantation and expansion of CAR T cells in patients with CLL, and enhanced its targeted cytotoxic activity.110 The outcomes could be attributed to downregulation of immunosuppressive receptors and improve in the proliferation and activation functions of T cells by ibrutinib.110,111 On the other hand, differentiation of Th2 cells could have been inhibited and immune response of Th1 cells could have been promoted by inhibiting the activity of IL-2 mediated T cell kinase.112 Other studies have shown superior effectiveness of the combined therapeutic approach than ibrutinib113,114 or CAR T-cell monotherapy,115 thus providing a new research direction to address the issue of resistance to CAR T-cell therapy.

Conclusion
In conclusion, advancements in our understanding of the mechanisms of resistance to CAR T-cell therapy are leading to new insights regarding this treatment. Novel strategies are being developed to overcome the resistance and improve clinical outcomes in patients with relapsed and refractory haematological malignancies. Various treatment approaches, such as targeting more than one antigen receptor, armoured CAR T-cells, fully human CAR T cells, CAR NK-cell therapy, and combination therapies with other immunotherapeutic agents are being explored to overcome the issue of resistance. However, the effectiveness of the aforementioned treatments remains unclear. Thus, further research is needed to maximise the duration of responses while minimising the risk of relapse.

Conflict of interest statement
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