

Chapter III.4

Clinical Potential of Antigen-Specific Therapies
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■ Abstract

In type 1 diabetes (T1D), pancreatic beta-cells are attacked and destroyed by the immune system, which leads to a loss of endogenous insulin secretion. The desirable outcome of therapeutic intervention in autoimmune diseases is the restoration of immune tolerance to prevent organ damage. Past trials with immune suppressive drugs highlight the fact that T1D is in principle a curable condition. However, the barrier in T1D therapy in terms of drug safety is set particularly high because of the predominantly young population and the good prognosis associated with modern exogenous insulin therapy. Thus, there is a general consensus that chronic

immune suppression is associated with unacceptable long-term safety risks. On the other hand, immune-modulatory biologicals have recently failed to confer significant protection in phase 3 clinical trials. However, the concept of antigen-specific tolerization may offer a unique strategy to safely induce long-term protection against T1D. In this review, we analyze the potential reasons for the failure of the different tolerization therapies, and describe how the concept of antigen-specific tolerization may overcome the obstacles associated with clinical therapy in T1D.

Keywords: type 1 diabetes · immune tolerance · HSP60 · DiaPep277 · oral antigen · nasal antigen · regulatory T cell

1. Immune suppression in T1D: the sledgehammer approach

At diagnosis, it is estimated that T1D patients possess only a limited pool of surviving beta-cells. Before diagnosis, the only consistent and reliable signs of ongoing autoimmunity are islet autoantibodies, but the pathophysiological processes at the level of the target organ remain incompletely characterized [1]. It is well known that T cells dominate the islet infiltrates [2], and that these cells are capable of killing beta-cells directly [3]. The beta-cells express high levels of MHC class I, and are therefore actively involved in their own demise. Autoreactive memory T cells also pose problems related to islet transplantation procedures, since these cells rapidly detect and de-

stroy any transplanted beta-cell mass [4]. Therefore, a genuine cure for T1D needs to tackle the immune component of the disease, and should preferably be aimed at the induction of long term tolerance.

Indeed, the combined outcomes of several trials using immune-suppressive agents such as cyclosporin [5] and azathioprine [6] have proven that blocking T cell function in T1D leads to beta-cell preservation. Cyclosporin, for instance, secures prolonged periods of insulin independence in many patients, which is a rigorous efficacy outcome measure [5, 7]. Unfortunately, maintenance of remission requires continuous treatment, and chronic cyclosporin therapy results in accelerated renal dysfunction [8]. Similarly, aggressive therapies such as autologous hematopoietic stem cell

transplantation involve similar risk-benefit considerations within the context of T1D [9]. It is therefore evident that this type of approach cannot be ethically justified for the prevention of T1D in young individuals at risk.

Abbreviations:

ADA – American Diabetes Association
 APC – antigen-presenting cell
 APL – altered peptide ligand
 CTL – cytotoxic T lymphocyte
 CTLA4 – cytotoxic T lymphocyte antigen 4
 DC – dendritic cell
 DIAPREVENT – Diabetes Prevention Trial
 DNA – deoxyribonucleic acid
 DPT-1 – Diabetes Prevention Trial 1
 GABA – gamma-aminobutyric acid
 GAD – glutamate decarboxylase
 GI – gastrointestinal
 GST – glucagon-stimulated
 HSP60 – heat shock protein 60 kDa
 ICA – islet cell antibody
 IFA – incomplete Freund's adjuvant
 Ig – immunoglobulin
 IL – interleukin
 INIT-II – Intranasal Insulin Trial II
 LADA – latent autoimmune diabetes of adulthood
 MMTT – mixed meal tolerance test
 MS – multiple sclerosis
 NCT – national clinical trial
 NOD – non-obese diabetic
 nTreg – natural Treg
 PDLN – pancreatic draining lymph node
 POINT – Primary Oral/Intranasal Insulin Trial
 T1D – type 1 diabetes
 TGF- β – transforming growth factor beta
 Th – T helper
 TLR2 – toll-like receptor 2
 TNF – tumor necrose factor
 Treg – regulatory T cell

2. Immune modulation in T1D: improved safety, reduced efficacy

To reduce the risks associated with chronic immune suppression, biologicals directed against defined immune receptors could be envisioned to tackle the immune system in a more specific fashion. Agents directed at the co-inhibitory receptor CTLA-4 [10] (abatacept), the B cell-specific protein CD20 [11] (rituximab), and the T cell signaling receptor CD3 [12, 13] (teplizumab/otelixizumab) have all been tested in T1D trials. For instance, anti-CD3 therapy, contrary to pharmacological immunosuppression, does not affect subsequent infectious or iatrogenic immunization to antigens that are not related to beta-cells. On the other hand, anti-CD3 agents do trigger transient reactivation of Epstein-Barr virus [14]. The hope is that

a short treatment course will trigger lasting tolerance, overcoming the need for chronic treatment which is afflicted with serious ethical considerations. Unfortunately, a recent series of immune modulation trials in new-onset T1D have been unable to permanently arrest the rate of C-peptide decline [10, 11, 15].

The rationale for the implementation of certain immune-modulatory biologicals in a recent-onset setting is not always evident. Non-mitogenic anti-CD3 agents are in fact the only class of biologicals that has consistently been able to reverse established hypoglycemia in several T1D models [16, 17]. Other agents such as CTLA-4Ig [18] and anti-TNF [19] only work in a prevention setting in animals, i.e. when given early and before the signs of overt hyperglycemia. It is therefore not entirely surprising that these approaches are unable to potentially prevent the C-peptide decline in a recent-onset setting in humans.

We conclude that the suboptimal effects achieved with immune modulation may reflect a need for higher doses or longer treatment durations. The projected risk-benefit ratio may then approach the profile seen with general immunosuppression.

3. The concept of antigen-specific tolerance

The overall goal of antigen-specific tolerance is to present known autoantigens to the immune system in such a way that they are seen as non-harmful, and consequently provoke a regulatory response. This is in fact exactly the opposite approach to that used by ordinary vaccinations. The regulatory response can consist of an attenuation or deletion of autoreactive T cells and/or the expansion and functional activation of regulatory T cells (Tregs). Various types of these Tregs exist, one of which was termed 'Th3' which specifically referred to mucosal-derived regulatory CD4 T cells producing mainly TGF- β [20]. We know that many of these Treg subtypes contribute to mucosal antigen-specific tolerance, and that their key phenotypic and functional features often overlap. The generation of antigen-specific Tregs is intended to regulate local inflammatory responses, i.e. within the pancreatic draining lymph nodes (PDLNs) and islets in T1D. In oral antigen therapies, the Peyer's patches are crucial immunological niches, since mice lacking these structures do not develop tolerance [21].

How do these antigen-specific Tregs regulate the entire autoreactive T cell repertoire? Here, a

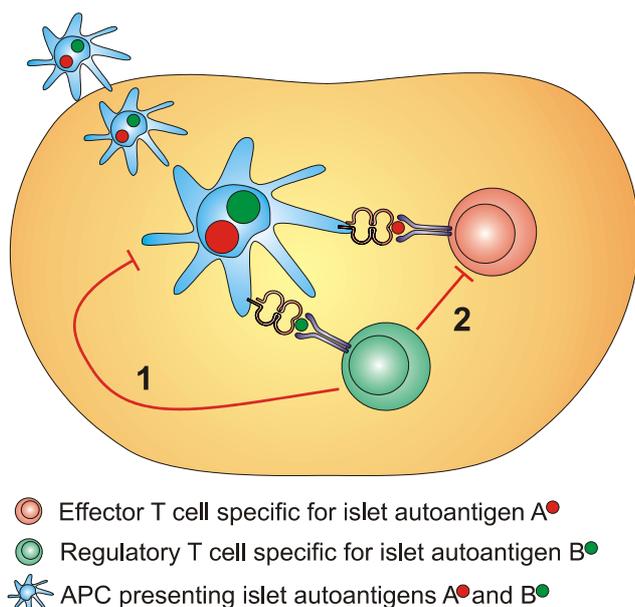


Figure 1. Antigen-specific tolerance and “linked suppression”. Antigen-specific regulatory T cells (Tregs) are raised under specific immunological conditions, for instance those that exist across the mucosal barrier. These Tregs may then regulate immune responses in the lymph nodes, for instance the pancreatic draining lymph nodes in T1D. Here, antigen-presenting cells (APCs) constantly drain the inflamed islets and shuttle islet antigens to the lymph nodes. The Tregs recognize the tolerizing antigens (here antigen A) and mediate immune suppression. The concept of “linked suppression” entails that any autoreactive T cell specific for another islet antigen (here antigen B) that is presented on the same APC will also be suppressed. Tregs mediate suppression via contact or cytokine expression on APC (1) or other T cells (2).

process termed linked suppression, originally stemming from transplantation studies, comes into play (**Figure 1**). Essentially, this concept entails that an antigen-specific therapy based on a single islet antigen can control autoimmune responses against other islet antigens, provided only these antigens are co-presented on the same dendritic cell (DC) as the tolerizing antigen. Thus, the actual ‘link’ in this concept is the local DC, which ensures that suppression only occurs at the inflammatory target site, and that unwanted suppression of normal immune responses does not occur. This type of ‘bystander suppression’ holds another therapeutic advantage, namely “operational capacity” even when the antigenic specificities of the other autoreactive T cells are unknown [22]. Treg

cells may orchestrate this bystander suppression by acting directly, through cytokine- or contact-dependent mechanisms or, more probably, indirectly on antigen-presenting cells (APCs) that in turn become tolerogenic. Recent evidence even shows that Tregs may be able to kill APCs directly, and as such prevent further T cell activation [23]. Since the concept of linked suppression enables to target the inter-individual variation of T1D patients with respect to their autoreactive T cell repertoire, this concept could aid in covering a broad spectrum of the population [2, 24]. Importantly, bystander tolerance (the term “infectious tolerance” is also used frequently) does not necessarily involve presentation of antigens by the same APC as in the linked tolerance model, since cytokine gradients can also limit tolerizing effects to a local environment.

Finally, do we have evidence that stimulating the more focused, localized action of antigen-specific Tregs may provide clinical benefit over increasing the numbers of peripheral natural Treg (nTreg) numbers? It appears that there is no significant overall difference in peripheral nTreg numbers between T1D patients and healthy controls [25]. This argues against the idea of simply increasing their numbers by adding more polyclonal nTreg to the immune system. Recent clinical trial data appear to confirm this notion. For instance, by combining rapamycin and interleukin (IL)-2, nTreg numbers can be boosted in T1D patients [26]. However, this correlated with a four-fold exacerbation in C-peptide decline compared with historical control subjects. Therefore, it seems that polyclonal nTreg expansion does not necessarily correlate with beta-cell preservation, and that antigen-specific Tregs may in fact be preferable.

4. Antigen-specific tolerance in T1D: excellent safety, but efficacy to be improved

Antigen-specific oral tolerance induction has reliably suppressed disease in a range of animal models for autoimmunity, including arthritis [27], multiple sclerosis [28], and T1D [29]. Other modes of mucosal (nasal [30, 31]) and non-mucosal (subcutaneous [32]) antigen delivery, alone or in combination with specific adjuvants, were explored, and promising data were obtained showing that antigen-specific tolerance induction can prevent the development of T1D. However, as actual reversal of established disease is typically not achieved via these antigen-based approaches this treatment

would be applicable in subjects at risk of T1D and possibly early-onset patients only. The overwhelming majority of studies revealed excellent safety profiles, with some notable exceptions in the diabetic BB rat model [33]. Due to significant cross-reactivity and endogenous mimicry effects [34] in adaptive responses, complete desired specificity of antigen-based intervention is not always guaranteed, and therefore should not be taken for granted.

Despite these robust data in animals, clinical studies in autoimmune disease patients have so far been less promising. In rheumatoid arthritis, collagen-specific tolerization therapy was tested in numerous trials, but with mixed outcomes [35]. Oral administration of myelin showed initial promise in multiple sclerosis [36], yet a subsequent phase 3 trial proved unsuccessful. Taken together, such trials probably highlight the notion that the outcome of antigen-specific therapy critically depends on a variety of factors, which also consistently influence treatment outcomes in animal models.

Looking at past experiences, we believe that the full potential of antigen-specific therapy for T1D has yet to be uncovered. Optimization of clinical translation could center on biomarker discovery and validation. We recently demonstrated that the presence of insulin autoantibodies prior to treatment with oral insulin/anti-CD3 combination therapy predicts efficacy in the NOD mouse [37]. To adequately apply potential immune biomarkers such as regulatory cytokine responses to islet antigens [38-40] or autoreactive T cell frequencies [41], in future trials, we need to better understand how these immune markers vary per individual during the natural course of T1D development and progression.

5. Examples of past and current antigen-specific therapy trials in T1D

5.1 Oral antigen administration

For obvious reasons, insulin is considered an important candidate for antigen-specific therapy in T1D. It is the only known beta-cell-restricted autoantigen as all other known protein targets are produced outside of the pancreas. Insulin is a major autoantigen in the NOD model [42], and evidence is mounting that insulin-reactive T cells play important roles in T1D [3]. Oral administration of whole insulin prevents autoimmune diabetes in spontaneous [29] and induced [43] animal

models. One conclusion from these animal studies is that the dose needs to be carefully chosen, as efficacy decreases in both the low and high end of the dosing spectrum [44]. Additionally, the origin of the insulin influences the efficacy, as optimal doses in mouse models differ for example between porcine and human insulin [45]. It also shows that a structurally intact whole antigen holds the most promise [27].

Most studies on oral insulin therapy in mice found an optimal dose within the single digit milligram range, given intra-gastrically twice weekly in a relatively large (~0.5 ml) volume of buffer. The Diabetes Prevention Trial (DPT-1) first screened over 100,000 relatives of T1D patients for islet cell antibodies (ICAs), and subsequently tested the effect of oral insulin in a secondary prevention setting [46]. Specifically, 7.5 mg of human insulin crystals was administered daily in non-entero-coated capsules or sprinkled over food. It was believed by the authors that the breakdown of insulin in the gastrointestinal (GI) tract was desirable to avoid any hypoglycemic effects. However, no beneficial outcome was reported, although post-hoc analysis showed a projected delay of 4.5 to 5 years in those patients with insulin autoantibody titers of over 80 nU/ml.

Many researchers in the field believe that some important factors could be altered to improve the efficacy of oral insulin in a prevention setting. First, the dose that can be extrapolated from mouse studies is several hundred folds higher than the one used in the DPT-1 trial. Given the importance of accurate dosing, as outlined above, this most likely is a pivotal variable. Second, insulin is extremely sensitive to denaturation and degradation, and it is unsure whether any naturally presented autoantigens will reach the relevant sites in the lower GI tract. Finally, this type of therapy may only work in patients with ongoing anti-insulin autoimmunity, and thus prior insulin autoantibody screening is required, as mouse studies appear to confirm [37]. A TrialNet study is underway to test this latter hypothesis (NCT00419562). All trials with oral insulin in a recent-onset setting, including the IMDIAB [47], Diabète Insuline Orale group [48], and the trial by MacLaren *et al.* [49] were unsuccessful. Importantly, all used low daily insulin doses and lacked any entero-protective formulation. Our studies using the *in silico* predictive platform developed by Entelos Inc. showed, at least for nasal insulin peptide tolerization, that low frequency administration, as done in mice, was more effective than high frequency dosing [50]. We conclude that oral insu-

lin therapy in T1D may still hold clinical potential, provided that parameters such as dosing, formulation methods, and inclusion criteria are optimized.

5.2 Nasal antigen administration

An alternative mode of mucosal administration, nasal insulin inhalation, was characterized in mouse models [30]. In contrast to the unpredictable bioavailability in the small intestine after oral administration, nasal administration could aid in better preserving the antigenic structure for optimal presentation to the mucosal immune system. A pilot study showed that nasal insulin treatment is safe and tends to induce immune changes consistent with the establishment of tolerance to insulin [51].

However, a large trial of daily nasal insulin in islet autoantibody-positive children at very high risk for type 1 diabetes found no effect on progression to diabetes [52]. A recent trial in new-onset patients also demonstrated no effect on C-peptide preservation, yet revealed promising immunological changes suggestive of tolerance induction [53]. A large nasal insulin trial (INIT-II, NCT00336674) in relatives of individuals with T1D is underway in Australia and New Zealand. The Pre-POINT trial which is currently being enrolled aims to identify optimal timing, disease stage, dose, and route of administration by intervening with oral or nasal insulin in genetically at-risk children before the appearance of islet autoantibodies [54].

5.3 Subcutaneous antigen administration

(Prepro)Insulin. Subcutaneous administration of insulin prior to diagnosis has been explored as a secondary prevention strategy, based on encouraging data in animal models [32] and pilot trials [55]. In addition to the effects on the immune system, it was hypothesized that insulin may act through metabolic effects, offering much needed 'rest' to the stressed beta-cells in at-risk individuals [56]. Recent data mechanistically support this assumption as beta-cells producing higher levels of endogenous insulin were shown to be more vulnerable to recognition and killing by preproinsulin-specific CTLs [3].

The DPT-1 study group assessed the outcome of parenteral insulin administration in a large cohort of high-risk individuals, and failed to demonstrate any effect on functional beta-cell preservation [57]. Alternative approaches include the use of insulin B chain administration in incomplete Freund's adjuvant (IFA) and proinsulin DNA vac-

ination [38, 39]. While some immunological evidence exists that supports their tolerogenic capacity, these therapies remain to be validated in large controlled trials.

An altered peptide ligand (APL) of insulin B9-23, an important autoantigen, was evaluated in a four-arm trial in recent-onset T1D. Patients were injected with 3 different doses or placebo at randomization, 2 weeks, 4 weeks, and thereafter monthly until 24 months. Maintenance of beta-cell function was not observed at any of the doses tested [58]. Since disease exacerbation with APL was seen in multiple sclerosis (MS), extreme caution is obviously advised in any future trials involving APL [59].

Glutamic acid decarboxylase (GAD). GAD is an enzyme that catalyzes the rate-limiting step in the biosynthesis of the inhibitory neurotransmitter GABA (γ -aminobutyric acid) [60]. The protein is broadly expressed in the nervous system and in other tissues such as the beta-cells. Two mammalian isoforms of GAD with different molecular weights were identified, GAD65 and GAD67, with GAD65 being the main immunogenic isoform in T1D [61]. GAD autoreactivity predicts clinical onset, is closely associated with recent-onset T1D [62], and thus represents an important diagnostic tool for clinicians. Patients suffering from a rare neurological condition called stiff man syndrome also exhibit GAD autoreactivity. Stiff-man syndrome is characterized by severe muscle stiffness with accompanying muscle spasms. A high prevalence of diabetes in patients with stiff man syndrome has been documented [63]. However, stiff man syndrome patients frequently show different GAD-specific T cell repertoires and distinct humoral responses (isotype) to GAD, suggesting fundamentally different underlying immunopathology in both diseases [64].

Not unlike insulin, GAD autoreactivity plays an essential role in the disease process in the NOD model [65]. A variety of tolerization therapies against GAD proved highly successful in NOD mice [31, 66, 67]. The treatment regimen was taken forward into controlled trials consisting of subcutaneous injections of GAD protein in aluminium hydroxide (alum), a common vaccine adjuvant. This adjuvant is known to skew Th1-dominated cellular immune responses, as in T1D, towards a Th2 humoral response [68]. An optimal dose of 20 micrograms (given twice, four weeks apart, C-peptide measurement at 24 weeks) was found in latent autoimmune diabetes of adulthood (LADA) patients, whereas both lower and higher doses

were inefficacious [69]. The beneficial effects on fasting C-peptide of the 20 microgram dosing were still measurable after five years, which indicates that long-term tolerance was established [70]. A phase II study, in a recent onset design, was then conducted, and showed improvement of C-peptide preservation, particularly in T1D patients with short disease duration, which was again maintained for at least 4 years [71, 72]. GAD65-specific Tregs [73], decreased antigen-specific Th1 responses [74], and T cell inhibitory pathways upon antigen stimulation [75] were all demonstrated in the GAD-treated patients.

Unfortunately, a recent-onset TrialNet study subsequently failed to confirm these positive outcomes [76]. Two phase 3 trials were recently conducted, one in Europe [77] and one in the U.S. (DIAPREVENT, NCT00751842), without significant evidence of beta-cell preservation. While much of the optimism for GAD-alum treatment for diagnosed T1D patients has vanished, an ongoing secondary prevention trial (DIAPREV-IT; NCT01122446) should provide more insight into the question of whether the drug can be used to protect high-risk individuals.

60 kDa heat-shock protein (HSP60, p277, DiaPep277). HSP60 has long been known as an autoantigen in the NOD mouse [78]. Specifically, a HSP60-derived peptide (p277) harbors the potential to protect mice from diabetes [79, 80], and has the ability to induce disease in non-susceptible strains after vaccination [81]. The protein appears to be a relatively unimportant autoantigen in patients, although some evidence exists that autoreactivity arises during the natural course of T1D [82].

The peptide used in clinical trials (DiaPep277, TEVA Pharmaceuticals) differs from the native p277 sequence in two amino acid positions that were introduced for stabilization purposes. A pilot study tested the efficacy of three subcutaneous 1 mg p277 injections in patients with recent-onset T1D [83]. Glucagon-stimulated (GST) C-peptide was determined at 10 months, and was higher in the DiaPep277 treated group, an effect that was sustained with follow-up to 18 months [84]. Mechanistically, DiaPep277 skews T cell reactivity to hsp60 and p277 towards a Th2 functional phenotype, but binds directly to toll-like receptor 2 (TLR2) on Tregs, which provokes better regulatory potential [85].

A number of other small trials with DiaPep277 were conducted, which all showed excellent safety, yet were not powered to offer conclusive evidence of efficacy [86-89]. The study by Huurman and co-

workers revealed that the proliferative T cell response to p277 is able to distinguish between treatment group and placebo, and may serve as a future biomarker [40]. These immune biomarkers need to be further developed, but the latter data suggest that efficacy, at least in part, correlates with an altered immune response against the immunizing antigen.

A successful phase 3 trial has now been completed, and data presented at the 2012 ADA Annual Sessions reported that C-peptide at 24 months showed an improvement with the glucagon stimulation test (GST), but not with the mixed meal tolerance test (MMTT). A press release also announced a reduction of hypoglycemia frequencies and insulin usage in the treatment group (<http://www.andromedabio.com/news.php>). A second phase 3 trial is underway, and has recently completed recruitment. The phase data support the hypothesis that antigen-specific therapies represent a safe and powerful approach, even during the advanced phases of disease. Interestingly, the dose used in the phase 3 trial (1 mg DiaPep277) was chosen based on reduced hypoglycemia frequencies, whereas the study by Huurman *et al.* showed that C-peptide preservation was most significant at a dose of 2.5 mg [86]. These results suggest that further dose optimization may lead to even better efficacy.

6. The future of antigen-specific therapy

Trials with immune-suppressive agents have taught us that tackling the immune-related component of T1D is an effective strategy to preserve functional beta-cell mass. The safety profile of these therapies, however, is unsatisfactory given the high quality of life that can be achieved with modern insulin therapy. Furthermore, we have come to the conclusion that long-term tolerance is preferable to short-term remission achieved by immunosuppression. New biologicals such as anti-CD3 have yet to meet the high expectations that were raised by animal studies and phase 2 trials. The phase 3 study with DiaPep277 in recent-onset T1D provides a glimmer of hope that antigen-specific tolerization could be used to treat late-stage disease at around the time of clinical diagnosis.

It is still puzzling why the impressive efficacy record of antigen-specific therapy in animal models does not translate to the clinic. To find answers, first it should be kept in mind that most antigen-specific therapies only work in a prevention

setting in animal studies. As outlined above, a combination of ethical considerations, shortened trial durations, and cost minimization requirements urge trial sponsors to prefer a recent-onset setting. Quite clearly, this setup implicates a high stringency for efficacy testing (end-stage disease), as compared to a secondary prevention setting, and offers a less meaningful endpoint (C-peptide) in comparison with the delay of diabetes. Additionally, current preclinical animal research focuses almost entirely on devising tolerogenic strategies for a naïve specific repertoire. However, the challenge for tolerogenic strategies in clinical autoimmune settings is to understand how regulation can be produced on the background of an ongoing chronic inflammatory response against the same antigens to which tolerance is to be produced. Ignoring this challenge may, for example,

explain the surprising deleterious effects of the rapamycin/IL-2 trial mentioned above.

Second, important variables such as antigen dose, formulation, and frequency of administration have often been characterized in detail in animal models, yet were largely ignored in the inception of clinical trials. Despite all their limitations, we argue that animal models should continue to contribute in making better informed decisions on the path to clinical translation. Third, suitable immune biomarkers are needed to better identify the correct target population, and to correlate immune function with clinical benefit. Finally, it is believed that for the adequate treatment of established disease, combination therapies are warranted, although generally more practical obstacles need to be overcome than with monotherapies [90].

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