

Targeting immune checkpoints in breast cancer: an update of early results



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ABSTRACT

The immune tumour microenvironment has been shown to play a crucial role in the development and progression of cancer. Expression of gene signatures, reflecting immune activation, and the presence of tumour-infiltrating lymphocytes were associated with favourable outcomes in HER2-positive and triple-negative breast cancer. Recently, immunotherapy with immune checkpoint blockade induced long-lasting responses and improved survival in hard-to-treat malignancies (ie, melanoma and non-small cell lung cancer) and are changing treatment paradigms in a variety of neoplastic diseases. Immune checkpoint blockade has been evaluated in breast cancer, particularly in the triple-negative subtype, with promising results observed in monotherapy or in combination with chemotherapy in the metastatic and neoadjuvant settings. However, identification of patients who are most likely to benefit from immune checkpoint blockade remains challenging, with many patients not responding to treatments and a significant financial cost. The combination of immune checkpoint blockade with conventional cancer treatments such as chemotherapy, radiotherapy, targeted therapies or with other immunotherapies is a promising strategy to potentiate its efficacy in breast cancer although further research is required to effectively identify who will respond to these immunotherapies. In this review we report the most recent results that emerged from trials testing immune checkpoint blockade and potential predictive biomarkers and emphasise the new strategies that are under clinical development in breast cancer.

INTRODUCTION

Cancer immunotherapy has historically been used in melanoma,¹ bladder and kidney tumours.² In these malignancies, the antitumour immune response was boosted with immune stimulants, such as interleukin-2,^{3,4} interferons (IFNs)⁵ and Calmette-Guérin Bacillus.⁶ These compounds represent the earliest forms of immunotherapy used in oncology to manipulate the immune system. Lately, a new form of passive immunotherapy using monoclonal antibodies (mAbs) targeted to tumour antigens (Ag) has been introduced in the clinic. Apart from blocking specific signalling pathways, mAbs are also able to stimulate immune responses through antibody-dependent cell-mediated cytotoxicity (ADCC).⁷ Two mAbs targeting

the human epidermal growth factor receptor (HER2), trastuzumab and pertuzumab, have significantly improved the outcomes of patients with HER2-positive breast cancer (BC),⁸ representing the first successful passive immunotherapeutic approach in BC.

Active immunotherapy using immune checkpoint blockade (ICB) represents a novel therapeutic approach for a variety of cancers, with promising activity. ICB uses mAbs targeting inhibitory immune checkpoints and has demonstrated impressive results in a variety of solid tumours and haematologic malignancies.^{9–18} Accordingly, some of these ICB drugs have been approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of melanoma, non-small cell lung cancer (NSCLC), urothelial carcinoma, renal cell carcinoma, head and neck cancer and classical Hodgkin lymphoma.

ICB functions by harnessing and enhancing the activity of the immune system by disrupting negative immune regulations to boost the antitumour immune response. Other immunomodulatory therapies potentiate costimulatory pathways or stimulate the innate immunity or interact with the immune suppressive tumour microenvironment. Additional alternative strategies in cancer immunotherapy include vaccines or cellular therapies, for example, with tumour-infiltrating lymphocytes (TIL)¹⁹ or autologous T cells genetically modified to express chimeric antigen receptors (CAR T cells).²⁰

BC was initially considered as a non-immunogenic tumour, but recent studies have demonstrated that the expression of immune-related genes and the presence of immune infiltrates in primary tumours were associated with a better clinical outcome,^{21–27} particularly in the most aggressive subtypes (HER2-positive and triple-negative (TNBC)). In addition, and consistent with their function, specific subsets of immune cells were correlated with outcome in BC. CD8⁺ T cells, usually representing cytotoxic T cells, are able

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to directly kill cancer cells and their presence was associated with a better prognosis.²⁸ By contrast, FOXP3⁺CD4⁺ regulatory T cells (Tregs) act mainly by mediating immune tolerance and their presence correlated with a poor prognosis.^{29,30}

The recent success of ICB in solid and haematological malignancies, together with the growing body of evidence on the prognostic/predictive role of the immune system in BC, encouraged the development of new immunotherapeutic strategies, some of which are currently undergoing clinical trial in BC. ICB agents targeting inhibitory molecules expressed on the surface of immune cells, such as cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1) or its ligand, the programmed death-ligand 1 (PD-L1) expressed by both tumour and immune cells, have been evaluated in several early-phase trials in BC.^{31–41} In the metastatic setting, these drugs showed promising results as single agents, with higher response rates (RRs) observed in the TNBC subtype,³² PD-L1-positive tumours^{32,33} and also in combination with chemotherapy (CT).^{35,38} More recent studies have revealed that the PD-1/PD-L1 blockade combined with CT in the neoadjuvant setting increases pathological complete response (pCR) rates with manageable safety profiles in TNBC and HER2-negative BC.^{39–41}

The aims of this review are to provide an overview of the results obtained with ICB in BC and to give insights into the most relevant immunotherapeutic approaches currently under clinical development in BC. We then discuss future developments and the challenges which should be overcome, such as the identification of biomarkers for patient selection and the rationales for the development of multi-modal treatment strategies to potentiate efficient immunotherapy in BC.

TARGETING INHIBITORY IMMUNE CHECKPOINTS

Multiple negative regulatory mechanisms can inhibit the antitumour immune response through the expression of immune checkpoint molecules by both immune and tumour cells. Under physiological conditions, these mechanisms control and prevent the development of auto-immunity, limiting the damage generated from excessive or chronic inflammation.⁴² In cancer, upregulation of immune checkpoints may reflect the occurrence of an ongoing immune response. Alternatively, the expression of immune checkpoints in tumour cells can also be driven by oncogenic pathways.⁴³ Activation of these inhibitory pathways protects neoplastic cells from immune system-mediated destruction. Therefore, immune checkpoint inhibitors, such as anti-CTLA-4, anti-PD-1 and anti-PD-L1, have been developed to bypass the immune checkpoint, with the aim of rescuing and enhancing the function of antitumour effector T cells.

In BC, PD-1/PD-L1 blockade monotherapy^{31–34,36,37} and also in combination with CT delivered positive outcomes in early-phase trials.^{35,38–41} Following these successful developments, a variety of strategies combining ICB

with other agents, including targeted therapies, radiotherapy (RT) or other immunotherapeutic drugs, are currently under evaluation at different stages of clinical development.

PD-1/PD-L1 immune checkpoint blockade

PD-1 and its ligand PD-L1 are the most widely investigated targets for ICB. PD-1 is a T cell inhibitory receptor that belongs to the CD28 superfamily, and upon binding with its ligands, it inhibits activated T cells and downregulates T cell response.

PD-L1 can be expressed by both tumour cells and immune cells and has been found to be expressed in multiple solid tumour types. In BC, PD-L1 expression correlates with hormone receptor negativity, higher histological grade, proliferation and TIL infiltration.⁴⁴ As such, PD-1/PD-L1 have been evaluated as therapeutic targets in a variety of trials, of which results are discussed below.

Single-agent activity of PD-1/PD-L1 blockade

ICB with PD-1 and PD-L1 inhibitors was first investigated in metastatic BC. Pembrolizumab is a high-affinity, highly selective, humanised immunoglobulin IgG4k mAb against PD-1. Pembrolizumab has been evaluated as monotherapy in a phase Ib trial, which included 32 patients with PD-L1-positive metastatic TNBC (KEYNOTE-012 trial).³¹ The rate of PD-L1 positivity (positivity in the stroma or >1% of positive tumour cells by immunohistochemistry (IHC)) equalled to 58% of the screened population (n=111) (table 1). Pembrolizumab was shown to provide long-lasting responses in heavily pretreated patients (46.9% had ≥3 previous lines of therapy). The RR reached 18.5%, with one complete response (CR) and four partial responses (PRs) (with two out of four PRs lasting more than 2 years). Survival stood at 22% after 2 years.⁴⁵ Rapid disease progression was observed in patients with more than two fold elevations in baseline lactate dehydrogenase (LDH) levels.

The use of pembrolizumab as monotherapy in metastatic TNBC was further evaluated in a phase II, single-arm multi-cohort study (KEYNOTE-086). Patients were split into three cohorts depending on their clinical treatment setting and PD-L1 status (table 1). A combined positive score (CPS) evaluating PD-L1 expression on tumour and immune cells was used to determine PD-L1 status and was positive in 62% of the tumours in cohort A³⁶ and in 58% of the screened tumours in cohort B.³⁷ Results have been reported for cohorts A and B, but not yet for cohort C. For the 170 previously treated patients enrolled in cohort A, RR was 4.7% regardless of PD-L1 positivity, with 1 CR and 7 PRs.³⁶ In subgroup analyses, objective response rate (ORR) was improved in patients with a low tumour burden, normal LDH at baseline and non-visceral disease and appeared independent of PD-L1 expression. Preliminary results for the first 52 untreated patients preselected based on PD-L1 expression enrolled in cohort B revealed an ORR of 23.1%, whereas stable disease (SD) (≥24 weeks) and PD were observed in 17% and 58% of the patients,

Table 1 Trials of PD-1/PD-L1 blockade as monotherapy in metastatic breast cancer

Reference	Drug	Phase	Patients	PD-L1 Positivity (%)	PD-L1 test	Patients evaluated (n) (total)	ORR (%)	Responses	Duration and survival	Grade 3/4 AEs prevalence (%)	Grade 3/4 AEs
KEYNOTE-012 (NCT02447003); Nanda <i>et al.</i> ³¹	Pembrolizumab (anti-PD-1)	Ib	TNBC (PD-L1 ⁺)	58	>1% TC or positivity in stroma (clone 22C3)	27 (32)	18.5	1 CR, 4 PR	mDOR: not reached (15.0 to >47.3 weeks) mPFS: 1.9 months mOS: 11.2 months	15.6	Anaemia, aseptic meningitis, lymphopenia, headache, colitis, hepatitis and fever. One death for disseminated intravascular coagulation.
KEYNOTE-086 (NCT02447003); Adams <i>et al.</i> ³⁶	Pembrolizumab (anti-PD-1)	II	TNBC (PD-L1 unselected) (cohort A)	62	>1 CPS (clone 22C3)	170	4.7	1 CR, 7 PR	mDOR: 6.3 months mPFS: 2 months mOS: 8.9 months	12	Diarrhea, fatigue, nausea and pneumonitis
KEYNOTE-028 (NCT02054806); Rugo <i>et al.</i> ³⁴	Pembrolizumab (anti-PD-1)	Ib	TNBC (PD-L1 ⁺) (cohort B)	58	>1% TC or positivity in stroma (clone 22C3)	52	23.1	2 CR, 10 PR	mDOR: 8.4 months PFS: 2.1 months	8	Back pain, fatigue, hyponatraemia, hypotension and migraine
KEYNOTE-028 (NCT02054806); Rugo <i>et al.</i> ³⁴	Pembrolizumab (anti-PD-1)	Ib	ER+/HER2- (PD-L1 ⁺)	19	>1% TC or positivity in stroma (clone 22C3)	25	12	0 CR, 3 PR	mDOR: 8.7 to >44 weeks	16	Autoimmune hepatitis, increased GGT, muscular weakness, nausea and septic shock
(NCT01375842); Schmid <i>et al.</i> ³³	Atezolizumab (anti-PD-L1)	Ia	TNBC	34	>5% IC (IC 2/3) (clone SP142)	112 (115)	10	3 CR, 8 PR	mDOR: 21.1 months mOS (resp.): not reached (31 March 2016) mOS (non-resp.) lived >6 weeks: 9 months	11	Adrenal insufficiency, neutropaenia, nausea, vomiting, decreased white blood cell count; G5 pulmonary hypertension event in a patient with an atrial septal defect
JAVELIN (NCT01772004); Dirix <i>et al.</i> ³²	Avelumab (anti-PD-L1)	Ib	ALL BC subtypes (PD-L1 unselected)	63.2 9.6	>1% TC >10% IC (clone 22C3)	153 (168)	4.8	1 CR, 7 PR	mDOR: 28.7 weeks		Fatigue, anaemia, increased GGT and autoimmune hepatitis and arthralgia, 2 treatment-related deaths (acute liver failure and respiratory distress)

AACR, American Association of Cancer Research; AE, adverse event; ASCO, American Society of Clinical Oncology; BC, breast cancer; CPS, combined positive score; CR, complete response; CT, chemotherapy; ER, oestrogen receptor; GGT, gamma-glutamyl transferase; IC, immune cell; JCO, *Journal of Clinical Oncology*; mDOR, median duration of response; mOS, median overall survival; mPFS, median progression-free survival; NR, not reported; ORR, objective response rate; PD, progressive disease; PR, partial response; RECIST, Response Evaluation Criteria In Solid Tumours; resp., responders; SABCS, San Antonio Breast Cancer Symposium; TC, tumour cell; TNBC, triple-negative breast cancer.

respectively.³⁷ Analysis of overall survival (OS) in cohort A demonstrated a benefit for responding patients.³⁶

Finally, pembrolizumab was also evaluated in a phase Ib trial (KEYNOTE-028), which included patients with PD-L1-positive advanced ER-positive/HER2-negative BC³⁴ (table 1). Nineteen per cent of the screened tumours were PD-L1 positive, and 25 heavily pretreated patients were included in the study. With a median duration of follow-up of 7.3 months, ORR was 12% with SD in four patients (16%) and PD in five patients (60%).

Alongside the PD-1-targeting pembrolizumab, several anti-PD-L1 therapies have been generated including the human IgG1-targeting mAb atezolizumab (MPDL3280A). Atezolizumab was tested as monotherapy in 115 patients with metastatic, PD-L1 unselected TNBC^{33 46} (table 1). Objective responses (ORs) were observed in 10% of the patients and were higher in patients receiving atezolizumab as a first-line treatment or in patients with high PD-L1 expression on tumour-infiltrating immune cells. mDOR was reported as 21.1 months, and median OS (mOS) (ranging from 4 to 37 months) was not reached in responders (n=15). Higher ORR and mOS were observed in patients whose tumours had >10% TIL and >1.35% intratumoural CD8⁺ TIL.

Avelumab, a fully human anti-PD-L1, IgG1 antibody was assessed as a single agent in 168 patients with unselected advanced or metastatic BC in the JAVELIN phase Ib trial (table 1). The ORR was modest (4.8%) with subgroup analyses demonstrating a higher RR in patients with TNBC (ORR: 8.6%). Interestingly, PD-L1 expression by tumour cells did not impact the efficacy of the treatment. The highest RR was observed in patients with TNBC with >10% PD-L1-positive tumour infiltrating immune cells (ORR: 44.4%) and mDOR was around 28 weeks.

The reported toxicity profile of single-agent PD-1 or PD-L1 blockade in BC was consistent with what has been previously reported in other malignancies, with fatigue, myalgia and nausea most commonly observed. A list of grade 3–4 observed toxicities is listed in table 1.

Numerous other trials are also underway to evaluate anti-PD-1 or anti-PD-L1 monotherapies (online supplementary table s1). The ongoing phase III KEYNOTE-119 study (NCT02555657) is comparing pembrolizumab alone with single-agent CT per investigator's choice in patients diagnosed with metastatic or locally advanced TNBC. Other ongoing studies include a phase II trial evaluating pembrolizumab in metastatic inflammatory BC (NCT02411656) and the phase I/II trial testing the anti-PD-1 PDR100 in solid tumours, including metastatic TNBC (NCT02404441).

Considering the huge number of ongoing studies evaluating anti-PD-1/PD-L1 agents in metastatic BC, this review cannot be exhaustive but discusses the main trials of ICB in BC.

Synergistic combinations with PD-1 and PD-L1 blockade

The combination of PD-1 and PD-L1 blockade with conventional cancer treatments is a promising strategy

to increase RR and clinical benefit from immunotherapeutic agents, with a demonstrated scientific rationale.⁴⁷ The outcomes and development of several trials are described below.

Combination with chemotherapy

It has often been argued that CT has immunosuppressive effects; however, several chemotherapeutic agents were demonstrated to induce immunogenic cell death (ICD), which relies on three processes: translocation of calreticulin to the cell-surface, release of the Toll-like receptor agonist HMGB1 and release of ATP into the extracellular milieu.⁴⁸ As a result, ICD promotes the activation of dendritic cells (DCs), the presentation of tumour-associated antigens and the production of inflammatory cytokines.⁴⁸ This process increases the immunogenicity of neoplastic cells and primes the immune system, by stimulating innate immune effectors and by inducing cytotoxic T cell responses.⁴⁹

In BC, ICD generated by CT was able to modulate the antitumour immune contexture, by increasing T cell infiltration⁵⁰ and improving the CD8/FOXP3 ratio.⁵¹ Furthermore, some cytotoxic agents are also able to inhibit the immune suppression exerted by specific subpopulations of immune cells, such as protumour Tregs, which are depleted by low-dose cyclophosphamide and paclitaxel,^{52 53} and myeloid-derived suppressor cells (MDSCs) whose levels can be decreased by gemcitabine and docetaxel.^{54 55} Combination of ICB with CT may therefore potentiate the antitumour immune response.

Numerous trials combining ICB with standard CT are ongoing in the metastatic setting as well as in early-stage BC.

The combination of pembrolizumab with eribulin mesylate was evaluated in a phase Ib/II study enrolling 89 patients with TNBC patients treated with less than three prior lines of CT for their metastatic disease³⁵ (table 2). The interim analysis reported at the 2016 San Antonio Breast Cancer Symposium revealed a 33.3% ORR (ranging between 27.3% in patients pretreated with one to two therapies to 41.2% in untreated patients) in the first 39 patients evaluated. SD was observed in 28.2% patients and the durable SD rate (SD lasting ≥24 weeks) was 7.7%. There were no differences in RRs based on PD-L1 expression (29.4% and 33.3% in the PD-L1-positive and PD-L1-negative cohorts, respectively). The most frequently reported adverse events (AEs) were fatigue (69%), nausea (51%), alopecia (36%), neutropaenia (36%) and peripheral neuropathy (28%). The addition of pembrolizumab to CT is also evaluated in a randomised phase III trial in first-line treatment of PD-L1-positive metastatic TNBC (KEYNOTE-355, NCT02819518) (online supplementary table s1).

Atezolizumab in combination with nab-paclitaxel was administered in a phase Ib study, in metastatic TNBC patients previously treated with less than three CT lines (87% had previous taxanes)³⁸ (table 2). Confirmed ORR was observed in 38% of patients, with higher RRs

Table 2 Trials of immune checkpoint blockade in association with chemotherapy or hormone therapy in metastatic breast cancer

Reference	Drug(s)	Phase	Patients	PD-L1 Positivity (%)	PD-L1 test	Patients evaluated (total)	ORR, %	Responses	Grade 3/4 AEs prevalence	Grade 3/4 AEs
PD-1/PD-L1 blockade plus chemotherapy										
(NCT02513472); Tolaney <i>et al.</i> ³⁵	Pembrolizumab (anti-PD-1) + Eribuline	Ib/II	Metastatic TNBC	43	>1 CPS (clone 22C3)	39 (89) 1 st line (17) 2 nd to 3 rd line (22)	33.3 41.2 27.3	1 CR, 12 PR NR NR	36% NR NR	Neutropaenia, fatigue. Serious AEs (all non-fatal) occurred in 36% of patients
(NCT01633970); Adams <i>et al.</i> ³⁸	Atezolizumab (anti-PD-L1) + Nab-Paclitaxel	Ib	Metastatic TNBC	NR	>1% TC or IC (clone SP142)	32 (32) 1 st line (13) 2 nd line (9) >3 rd line (710)	38 46 22 40	NR NR NR NR	41% NR NR NR	Neutropaenia
CTLA-4 blockade plus hormone therapy										
Vonderheide <i>et al.</i> ⁹⁶	Tremelimumab (anti-CTLA-4) + Exemestane	I	Metastatic BC (ER+)	NA	NA	26	0	1 SD	There were no G4 AEs	Treatment-related serious AE: diarrhoea, fever and dehydration
Others										
(NCT00349934) Brignone <i>et al.</i> ¹⁰¹	IMP321 (recombinant soluble LAG3lg) + Paclitaxel	I	Advanced CT untreated (87% ER+, HER2-)	NA	NA	30 (33)	50	15 PR	18	Asthenia, neuropathy, allergic reaction and neutropaenia, appendicitis, Staphylococcus infection
(NCT02614833); Duhoux <i>et al.</i> ¹⁰²	IMP321 (recombinant soluble LAG3lg) + Paclitaxel	IIb	Metastatic BC (ER+/- HER2-)	NA	NA	15	NR	NR	NR	One cytokine release syndrome

ASCO, American Society of Clinical Oncology; AE, adverse event; BC, breast cancer; CR, complete response; ER, oestrogen receptor; IC, immune cell; mAb, monoclonal antibody; NR, not reported; NA, not applicable; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumours; SABCS, San Antonio Breast Cancer Symposium; SD, stable disease; SAE, serious adverse event; TC, tumour cell.; TNBC, triple-negative breast cancer.

in patients treated in the first line setting compared with second and third lines (46%–22% and 40%, respectively). The most common AE reported was decreased neutrophil count (53% all grade; 41% grade 3–4) and no treatment related deaths occurred. Considering the encouraging results of this combination, IMpassion130, a randomised phase III study, was initiated and is currently underway, evaluating nab-paclitaxel with or without atezolizumab as a first line treatment in metastatic TNBC (NCT02425891) (online supplementary table s1). PD-L1 expression is not an inclusion criterion, but responses will be stratified accordingly.

Some other ongoing trials currently evaluating anti-PD-1/PD-L1 agents in association with CT in the metastatic setting are listed in online supplementary table s1.

Combination with radiotherapy

RT is mainly used to control and eradicate local disease; however, it is also known to induce responses observed distant from the irradiated volume, defined as the abscopal effect by Dr Mole in 1953.⁵⁶ This abscopal effect following RT may be mediated by various immunologic mechanisms, such as ICD-releasing danger signals able to recruit and activate DCs or by inducing the production of immunostimulatory cytokines priming the antitumour immune response.^{57–59} Although abscopal responses remain relatively rare, they have been described in several types of cancer, including melanoma, lymphoma and adrenal cell carcinoma.⁶⁰ In less immunogenic tumours, such as BC,⁶¹ RT can be used to enhance immunostimulatory signals to improve the response to ICB.⁶² Several ongoing early-phase trials are evaluating this approach in BC (online supplementary table s1). Pembrolizumab is administered in combination with radiosurgery in a pilot study treating patients with oligo-metastatic BC in the absence of visceral metastases in the liver or in the brain (BOSTON II, NCT02303366); in a phase I study of advanced solid tumours (NCT02608385); or in association with hypofractionated RT in metastatic BC (RADVAX, NCT02303990); and also in association with palliative RT in HR-positive HER2-negative metastatic BC (NCT03051672) (online supplementary table s1). In metastatic TNBC, two phase II trials are evaluating RT plus ICB: one with pembrolizumab plus fractionated RT (NCT02730130) and another with nivolumab after a hypofractionated induction RT or low-dose CT (TONIC, NCT02499367). Results are yet to be reported.

Combination with targeted monoclonal antibodies

As targeted therapies with mAb are also able to stimulate immune responses through ADCC, their combination with ICB represents another attractive strategy with potential synergism.^{7 63} Preclinical models showed that ICB with anti-PD-1 was able to potentiate the immune-mediated effects of trastuzumab.⁶⁴

Various trials are evaluating anti-PD-1 or anti-PD-L1 agents in combination with anti-HER2-targeted therapies (trastuzumab or ado-trastuzumab emtansine) in

HER2-positive BC. The main studies are listed in online supplementary table s1.

Vascular endothelial growth factor is another target for mAbs in BC that can be inhibited through the use of the antiangiogenic mAb bevacizumab. Treatment with bevacizumab inhibited the infiltration of immune suppressive cells (ie: Tregs, macrophages, MDSCs) in a BC xenograft model.⁶⁵ Moreover, a clinical study in patients with renal cell carcinoma observed synergistic therapeutic effects for ICB with bevacizumab.⁶⁶ In HER2-negative metastatic BC, a pilot study is currently investigating the combination of durvalumab with bevacizumab in patients that progressed after treatment with bevacizumab alone (NCT02802098) (online supplementary table s1).

Combination with DNA-demethylating agents

Epigenetic dysregulation is now recognised as a common phenomenon involved in cancer initiation and progression. Aberrations induced by epigenetic mechanisms including DNA methylation, histone modifications, nucleosome positioning and non-coding RNA expression have been shown to be reversible and can be modulated by a variety of agents.⁶⁷ Recent reports have demonstrated that DNA-demethylating agents improve the immunogenicity and immune recognition of cancers cells. They upregulate immune signalling by the induction of an IFN response through the activation of endogenous retroviruses.^{68 69} Preclinical models combining hypomethylating agents and histone deacetylase inhibitors with ICB support the immunomodulatory activities of these agents and demonstrated improved antitumour activity.^{70 71}

Azacitidine, a reversible inhibitor of DNA methyltransferase that blocks DNA methylation, is under investigation in a phase II study evaluating its association with durvalumab in metastatic ER-positive/HER2-negative BC (NCT02811497) (online supplementary table s1). A further open-label phase I/II study of azacitidine in combination with pembrolizumab and the indoleamine 2,3-dioxygenase (IDO) inhibitor epacadostat will evaluate safety, tolerability, effects on the tumour microenvironment and efficacy in subjects with advanced solid tumours (ECHO-206, NCT02959437). An additional two cohort open-label phase II study is currently recruiting participants with locally advanced HER2-negative BC as candidates for short-term neoadjuvant immunotherapy with pembrolizumab and the hypomethylating agent decitabine (a DNA methyltransferase inhibitor), followed by standard neoadjuvant CT (NCT02957968).

RRx-001, an agent able to inhibit both methyltransferase and histone deacetylase, is under evaluation in combination with nivolumab in advanced solid tumours (PRIMETIME, NCT02518958).

Combination with endocrine therapy

Experimental BC models revealed that estradiol can mediate immune effects through various mechanisms such as enhancing macrophage influx *via* the release of chemokines.⁷² In addition, antioestrogen therapy

decreases antitumour immune response by inhibiting the generation of cytotoxic effector cells and the functional differentiation of DCs or by inducing Tregs.^{72–76}

In humans, tamoxifen induced *ex vivo* FOXP3 expression on TIL,⁷³ while the aromatase inhibitor letrozole was able to reduce the presence of intratumoural FOXP3⁺ Tregs.⁷⁷ A poor response to aromatase inhibitors was observed in patients with higher baseline expression of an inflammatory gene expression signature.⁷⁸ In responders to neoadjuvant anastrozole, it was observed an increased expression of genes related to inflammatory processes, with enrichment of those promoting T cell anergy.⁷⁹ The immune effects of anastrozole were also described in rat models, with increased levels of proinflammatory cytokines and suppression of Treg differentiation induced by this drug.⁸⁰

A combination approach with pembrolizumab in association with anastrozole, exemestane or letrozole (in the ER-positive cohort of the study) is being evaluated in patients with ER-positive metastatic BC (NCT02648477) (online supplementary table s1).

Other combinations

Anti-PD-1/PD-L1 agents are being evaluated with multiple immunomodulatory approaches or with other strategies to maximise patient response and benefit. ICB has been combined with agents targeting the immunosuppressive microenvironment. Pembrolizumab with epacadostat, an IDO inhibitor, was shown to be safe in patients with advanced solid tumours, including TNBC in the phase I/II ECHO-202/KEYNOTE-037 trial (NCT02178722) recently presented at the American Society of Clinical Oncology (ASCO) Annual Meeting 2017⁸¹ (online supplementary table s1). Combination of atezolizumab with an antagonist of the immunosuppressive adenosine-A2A receptor (NCT02655822) demonstrated antitumour activity in solid tumours, including metastatic TNBC⁸² (online supplementary table s1).

Phosphoinositide 3-kinase (PI3K) inhibitors, or AKT inhibitors, induced decreased expression of PD-L1 in TNBC cell lines, suggesting a link between phosphatase and tensin homolog, PI3K and the regulation of PD-L1 expression.⁸³ The phase I trial NCT02646748 is evaluating pembrolizumab in combination with Janus kinase inhibitor (JAK-1) or with a PI3K- δ inhibitor in TNBC.

Combination therapy with a mitogen-activated protein kinase (MEK) inhibitor (cobimetinib) and the anti-PD-L1 atezolizumab gave rise to 17% ORR in microsatellite stable colorectal cancer and was shown to increase major histocompatibility complex (MHC) class I expression and CD8⁺ T cell infiltration.⁸⁴ Phase I and II trials in BC are testing the combination of cobimetinib with taxane-based CT and atezolizumab (NCT02322814) as first-line treatment in metastatic TNBC or cobimetinib together with nivolumab and ipilimumab in TNBC (NCT01928394).

Poly (ADP-ribose) polymerase (PARP) inhibitors interfere with the mechanisms of DNA repair, increasing DNA damage that in consequence could lead to a higher

mutational burden and neoantigen expression, but also to the induction of hypoxia, which has been shown to selectively upregulate the expression of PD-L1 by protumour MDSCs.⁸⁵ A phase I study evaluated a PARP inhibitor (olaparib) in association with durvalumab (MEDI4736), a PD-L1 inhibitor in patients with metastatic TNBC and ovarian cancer and demonstrated that this combination was safe and active (NCT02484404).⁸⁶ Additionally, pembrolizumab is being evaluated with niraparib in advanced TNBC or recurrent ovarian cancer in a phase I/II study (KEYNOTE-162, NCT02657889). Other combinations under development are listed in online supplementary table s1.

PD-1/PD-L1 blockade in the neoadjuvant and adjuvant settings

Several trials are ongoing with anti-PD-1 and anti-PD-L1 agents in the (neo)adjuvant setting. The association between TIL infiltration and response to neoadjuvant CT has been suggested to rely on immune mechanisms, such as the ICD induced by cytotoxic agents. In this setting, addition of an immunomodulator could potentially activate or potentiate the antitumour response and increase the immune stimulation induced by CT, in order to achieve higher RRs and memory immune responses to prevent relapse. Compared with the adjuvant, in the neoadjuvant setting the tumour can be exploited as a source of antigens. As such in mice, increased efficacy of neoadjuvant *versus* adjuvant immunotherapy has been observed.⁸⁷

The I-SPY 2 randomised phase II trial investigated the impact on pCR rate following treatment with the combination of pembrolizumab with standard neoadjuvant CT (paclitaxel followed by doxorubicin and cyclophosphamide) in high risk TNBC and ER-positive/HER2-negative BC (NCT01042379)³⁹ (table 3). In preliminary analyses, the addition of pembrolizumab increased the likelihood of pCR achievement compared with paclitaxel, particularly in the TNBC subgroup. The phase Ib KEYNOTE-173 trial investigated the addition of pembrolizumab to neoadjuvant CT in locally advanced TNBC (NCT02622074)⁴¹ (table 3). Pembrolizumab was given in monotherapy before and then in association with CT (nab-paclitaxel followed by doxorubicin and cyclophosphamide, cohort A; or with carboplatin and paclitaxel followed by doxorubicin and cyclophosphamide in cohort B). Preliminary results presented at ASCO 2017 were promising, with higher pCR rates following both schemes of administration. MEDI4736, an anti-PD-L1, is currently under evaluation in a neoadjuvant phase I/II trial in combination with nab-paclitaxel and dose-dense doxorubicin plus cyclophosphamide for stage I–III TNBC (NCT02489448) (online supplementary table s1). Preliminary analysis of the phase I study showed that the combination was safe and well tolerated. Final phase I toxicity and efficacy on pCR results are awaited.

The use of ICB in conjunction with CT as neoadjuvant treatment is safe and well tolerated, with preliminary results demonstrating promising efficacy. Nevertheless,

Table 3 Results from trials of immune checkpoint blockade in the neoadjuvant setting

Reference	Drug(s)	Phase	Breast cancer subtype	Patients (n)	Responses
PD-1/PD-L1 blockade plus chemotherapy					
I-SPY-2 (NCT01042379); Nanda <i>et al.</i> ³⁹	Pembrolizumab with neoadjuvant paclitaxel followed by doxorubicin and cyclophosphamide	II	TNBC and ER+/HER2- BC	69	pCR rate: in TNBC 60% <i>versus</i> 20%; in ER-positive/HER2-negative 34% <i>versus</i> 13%
KEYNOTE-173 (NCT02622074); Schmid <i>et al.</i> ⁴¹	Pembrolizumab in association with nab-paclitaxel --> doxorubicin plus cyclophosphamide (cohort A)	Ib	Locally advanced TNBC	10	ORR: 80%; ypT0 ypN0 pCR rate: 50%
	Pembrolizumab in association with carboplatin plus nab-paclitaxel --> doxorubicin plus cyclophosphamide (cohort B)			10	ORR: 100%; ypT0 ypN0 pCR rate: 90%
CTLA-4 blockade					
McArthur <i>et al.</i> ⁹⁸	Ipilimumab with cryoablation	I	Operable BC, before undergoing mastectomy	19	Increase in circulating T helper (Th)1-type cytokines, activated ICOS ⁺ and proliferating CD4 ⁺ and CD8 ⁺ T lymphocytes

ASCO, American Society of Clinical Oncology; BC, breast cancer; ER, oestrogen receptor; ORR, objective response rate; pCR, pathological complete response; TNBC, triple-negative breast cancer.

the results of phase III randomised trials are awaited to confirm the benefit of these combinations in this setting.

Two randomised phase III trials are currently evaluating the combination of anti-PD-1/PD-L1 agents with neoadjuvant CT in TNBC (online supplementary table s1). In the KEYNOTE-522 trial, pembrolizumab is being given with taxane-based and anthracycline-based neoadjuvant CT and alone for nine cycles after surgery (NCT03036488). In the second trial, atezolizumab is being evaluated with nab-paclitaxel before surgery, followed by an anthracycline-based CT administered in the adjuvant setting (NCT02620280). Another phase III trial is randomising adjuvant pembrolizumab in patients with TNBC with residual disease at surgery after neoadjuvant CT (NCT02954874).

The combination of anti-PD-1 and endocrine therapy in early-stage BC is also under evaluation in two clinical trials. A phase II trial is testing durvalumab plus an aromatase inhibitor (exemestane) for 6 months before surgery in patients with early-stage ER-positive BC, whose tumours increased in CD8⁺ TIL levels after 6 weeks exposure to tremelimumab or other potentially immune-attractant agents (ULTIMATE, NCT02997995) (online supplementary table s1). Another phase II study is investigating the addition of pembrolizumab to endocrine therapy in localised inflammatory BC not achieving pCR after neoadjuvant CT (NCT02971748).

Biomarkers of response to PD-1/PD-L1 blockade

The success of ICB in a variety of solid tumours was achieved in a subset of patients only, highlighting the strong need for identifying reliable biomarkers predictive of benefit from these new treatments. PD-L1 expression by IHC on tumour cells was evaluated in a phase I trial testing pembrolizumab in advanced solid tumours.⁸⁸ None of the patients with PD-L1-negative tumour cells responded to the treatment, while 36% of patients with PD-L1-positive tumours experienced an OR. The predictive value

of PD-L1 expression was further demonstrated in several trials, but its use as a biomarker is still controversial.⁸⁹ Patients with PD-L1 negative tumours were excluded from many trials, making it difficult to properly define its predictive significance. Moreover, different antibodies, staining platforms and thresholds of positivity on different types of cells have been used across the various clinical trials to assess PD-L1 expression⁹⁰ (tables 1 and 2).

In BC, PD-L1 expression by IHC was used as inclusion criterion for some^{31 34 37} but not all trials of immunotherapy with anti-PD-1/PD-L1 agents.^{32 33 35 36 38-41} Differences in PD-L1 positivity thresholds and prevalences across BC trials are listed in tables 1 and 2. The KEYNOTE-012 trial showed that increasing expression of PD-L1 correlated with a higher probability of response (p=0.028 for ORR) and a reduction in the hazard for PFS (p=0.012) in this PD-L1-positive TNBC cohort treated with pembrolizumab.³¹ PD-L1 positivity was also associated with higher RRs in patients treated with atezolizumab³³ and avelumab.³² In contrast, in the phase II KEYNOTE-086 trial, there was no difference in ORR according to PD-L1 expression in cohort A of patients with TNBC.³⁶ In the study by Tolaney *et al.*,³⁵ PD-L1 expression was not predictive of response in this cohort of TNBC patients treated with pembrolizumab in association with eribulin mesylate.

The interrogation of multiple biomarkers including PD-L1 expression could potentially improve prediction of the disease course and guide immune anticancer therapy. A model combining PD-L1 expression with TIL levels has already been proposed to classify tumour microenvironments in order to discriminate tumours that are most likely to respond to PD-1/PD-L1 blockade.^{91 92} The recent update of the phase I study evaluating atezolizumab in metastatic TNBC revealed that patients with high TIL and CD8⁺ cells benefit most from the treatment.³³ Interestingly, in this study, immune infiltration was a more

robust predictor of benefit than PD-L1 expression by immune cells. Other studies are investigating TIL in trials of immunotherapy in BC.

Circulating LDH was associated with a reduced benefit to ICB *via* anti-PD-1 in melanoma.⁹³ Similarly, in patients with TNBC treated with pembrolizumab, elevated baseline circulating LDH was associated with rapid disease progression in the KEYNOTE-012 trial³¹ and with lower RRs in the KEYNOTE-086 trial.³⁶ Therefore LDH may be a useful biomarker, guiding treatment decisions with anti-PD-1/PD-L1 agents in metastatic TNBC.

CTLA-4 blockade

In BC, ICB with anti-CTLA-4 agents has been given in combination with other therapeutic approaches including endocrine therapy (table 2) and anti-PD-1/PD-L1 agents in the metastatic setting and with cryotherapy in early-stage BC (table 3). CTLA-4 and PD-1 receptors mediate immune inhibition by two non-redundant and complementary pathways.^{94 95} Of note, in melanoma, dual CTLA-4 and PD-1 ICB increased RRs and durable responses compared with single agents, although with a significant increase in toxicity.⁹

The fully human IgG2 anti-CTLA-4 mAb tremelimumab was administered as immune cell attractant in association with the aromatase inhibitor exemestane in a phase I trial including 26 patients with ER-positive metastatic BC⁹⁶ (table 2). Although no ORs were achieved, SD lasting for more than 12 weeks was observed in 42% of the patients. The combined treatment reduced the presence of circulating Tregs and increased the number of circulating activated effector T cells. The most common treatment-related AEs were: diarrhoea (46% of patients), pruritus (42%), constipation (23%) and fatigue (23%). A phase II ongoing trial, ULTIMATE (NCT02997995), is using tremelimumab as immune attractant in the neoadjuvant setting as mentioned above. Dual ICB with the anti-CTLA-4 tremelimumab and the anti-PD-L1 durvalumab is being tested in two trials (NCT01975831 and NCT02536794) in advanced non-TNBC and HER2-negative BC, respectively. Preliminary results on the NCT01975831 study presented at ASCO 2017 revealed that the combination of tremelimumab and durvalumab has a manageable safety profile. In the cohort of non-TNBC (n=10 patients), 2 SD and 1 PR were observed.⁹⁷

Ipilimumab is a recombinant human IgG1 mAb against CTLA-4 that has been investigated in BC in association with cryoablation in patients with operable BC, before undergoing mastectomy⁹⁸ (table 3). This treatment approach was safe, with only one grade 3 AE reported (rash after ipilimumab) that did not delay preplanned surgery. Combination of cryoablation and ipilimumab lead to an increase in circulating T helper (Th)1-type cytokines, activated ICOS⁺ and proliferating CD4⁺ and CD8⁺ T lymphocytes. Interestingly cryoablation +/- ipilimumab modelled the clonal repertoire of intratumoural T cells, with patients that received the combination approach demonstrating greater levels of

peripheral blood and intratumoural T cell clones after therapy.⁹⁹

Some of the ongoing trials evaluating CTLA-4 blockade in combination with other ICB agents are listed in online supplementary table s1.

Targeting LAG3 and TIM3

Immunotherapeutic approaches targeting other inhibitory immune checkpoint molecules, such as lymphocyte-activation gene 3 (LAG3) and T cell immunoglobulin and mucin domain-containing molecule 3 (TIM3) are in development.¹⁰⁰ However, drugs interacting with these receptors are usually given in combination with anti-PD-1/PD-L1 agents, rather than as monotherapy, as they target alternative, non-redundant inhibitory pathways in T cells.

LAG3 is an inhibitory receptor that binds to MHC-II molecules expressed on the surface of antigen presenting cells (APC), preventing their activation. A mAb directed against LAG3, BMS-986016, is being tested alone or in combination with nivolumab in a phase I trial enrolling patients with advanced solid tumours (NCT02966548) (table 2). A recombinant soluble LAG3-Ig fusion protein, IMP321, binds to MHC-II molecules mediating APC activation and promoting CD8⁺ T cell activation. IMP321 was evaluated together with weekly paclitaxel in 30 patients diagnosed with advanced HER2-negative BC not previously treated with CT for the metastatic disease.¹⁰¹ This combination was well tolerated and resulted in an ORR of 50%, with 90% of the patients demonstrating a 6-month clinical benefit (table 2). A randomised phase IIb trial is underway assessing the combination of first-line weekly paclitaxel plus IMP321 in ER-positive/HER2-negative metastatic BC (NCT02614833) (table 2, supplementary table s1). This combination was shown to be safe in the 15 patients treated, with a grade 1 cytokine release syndrome observed in one patient.¹⁰²

TIM3 is an inhibitory receptor that binds to its main ligand galectin-9, promoting the death of IFN-gamma-producing, Th1-type CD4⁺ T cells. A phase I-Ib/II open-label multi-centre, first-in-human study of the anti-TIM3 MBG453 as single agent or in association with the anti-PD-1 PDR001 is currently recruiting participants with advanced solid tumours (NCT02608268) (online supplementary table s1).

TARGETING COSTIMULATORY PATHWAYS

Another approach to enhance the antitumour response is to target costimulatory immune checkpoint molecules that are expressed by activated lymphocytes.⁴⁷ Binding of these molecules to their ligands expressed on APC provides signals promoting T cell activation, expansion and differentiation after antigen recognition.

OX40 (CD134) is a costimulatory molecule that can be expressed by activated T cells and Tregs.^{103–106} OX40 is an interesting pathway for immunotherapy, as ligation to its

ligand promotes expansion, differentiation and survival of effector T cells, while inhibiting Treg function.^{101–103}

MEDI6469, MEDI6383 and MEDI0562 are agonistic mAbs that bind to OX40 (CD134) and are being tested in metastatic BC, alone (NCT02205333, NCT02221960, NCT02318394) or in combination with ICB (with tremelimumab or durvalumab in NCT02205333; with durvalumab in NCT02221960) or with RT (online supplementary table s1). MEDI6469 is being given in association with stereotactic body RT on liver or lung metastases (NCT01862900) (online supplementary table s1).

4-1BB is another costimulatory immune checkpoint molecule expressed by activated T cells, binding to 4-1BBL on the surface of activated APC. This interaction stimulates and expands effector T cells, also potentiating cytokine production.^{107–108} In addition, 4-1BB enhances the cytotoxicity mediated by NK cells.¹⁰⁹ A humanised agonistic mAb targeting the 4-1BB, urelumab is being tested in phase I and I/II trials of advanced solid tumours, alone (NCT00309023) or in combination with nivolumab (NCT02253992 and NCT02534506) (online supplementary table s1).

FUTURE CHALLENGES OF IMMUNE CHECKPOINT BLOCKADE IN BREAST CANCER

The most relevant challenges in cancer immunotherapy include: the optimisation of patient selection through the identification of reliable predictive biomarkers of response to these treatments^{110–111} and the evaluation of responses through the use of criteria specific for immunotherapeutic agents.¹¹²

Response to ICB has been associated with specific intrinsic and extrinsic properties of tumours or of the host that have been recently classified as the elements of the cancer-immune set point.¹¹¹ Intrinsic properties reflect the degree of tumor foreignness,¹¹⁰ linked to the mutational burden and presence of neoantigens that can be recognised by the immune system, as shown in NSCLC and melanoma.^{113–114} Foreignness of breast tumours might vary by molecular subtype. Higher number of mutations was observed in the HER2-enriched (2.05 mutations per Mb) and basal-like (1.68 mutations per Mb) followed by the luminal B (1.38 mutations per Mb) and luminal A (0.84 mutations per Mb) BC molecular subtypes.¹¹⁵

In addition to the intrinsic properties of the tumours, extrinsic factors, such as exposure to sunlight and to cigarette smoke, the presence of viral infections and the composition of the gut microbiota, were classified as elements of the cancer-immune set point.¹¹¹ The exposure to sunlight and cigarette smokes was relevant for melanoma and NSCLC, respectively, while the presence of viral infections might impact the response to ICB in Human Papilloma Virus positive tumours and Epstein-Barr Virus related tumours. Preclinical evidence showed that several *Bacteroides* and *Bifidobacterium*

species influenced the efficacy of ICB with anti-CTLA-4 and anti-PD-L1 mAb in mice.^{116–118} The role of the gut microbiota in patients with BC treated with ICB requires further investigations.

Evaluation of responses to immunotherapeutic agents represents an additional challenge for oncologists and radiologists. The biological mechanisms and timing of responses to ICB are different from previously described chemotherapeutic and targeted agents, with unique patterns observed (ie: pseudoprogressions, hyperprogressions, delayed and durable responses). Aside from traditional primary endpoints, SD, DOR, pseudoprogression followed by durable responses and OS should be weighted in a different way when faced with ICB. Noteworthy, in the phase I trial presented at the American Association of Cancer Research Meeting in 2017,³³ pseudoprogression was observed in patients with metastatic TNBC treated with atezolizumab having PD by Response Evaluation Criteria in Solid Tumours (RECIST) v.1.1. Recently updated guidelines for the use of modified RECIST (iRECIST) in trials of immunotherapies were published in efforts to standardise and validate these criteria and harmonise the interpretation of the results.¹¹²

LDH levels, PD-L1 expression (although controversial) and TIL have demonstrated a predictive value in BC patients treated with PD-1/PD-L1 blockade. The cancer immunogram summarised some candidate biomarkers that have been studied in melanoma and NSCLC.¹¹⁰ The general individual immune status, mirrored by the levels of circulating lymphocytes and the neutrophil to lymphocyte ratio (NLR) and the increase of the C reactive protein, the erythrocyte sedimentation rate and LDH, were shown to influence the response to ICB. In patients with melanoma treated with pembrolizumab, high baseline relative eosinophil count and high baseline relative lymphocyte count, low LDH and the absence of metastases other than soft tissues/lung were associated with favourable OS.⁹³ Furthermore, the presence of reactive neoantigen-specific intratumoural T cells within the tumour microenvironment¹¹⁴ and the expression of PD-L1^{110–119} represent further putative predictive biomarkers.

Four assays are registered with the US FDA for PD-L1 assessment by IHC, but harmonisation of PD-L1 evaluation by pathologists is needed. A prospective, multi-institutional study was conducted in NSCLC to compare the performance of the four tests, revealing that one in particular underestimated the expression of PD-L1. Pathologists were more concordant when evaluating PD-L1 expression by tumour cells, compared with PD-L1 expression by immune cells.¹²⁰ Nevertheless, in BC the predictive role of PD-L1 is still under debate, with contrasting results.^{32–33–35–36} Immuno-Positron Emission Tomography (PET) imaging might represent an additional tool for the evaluation of PD-L1 expression, as shown by Bensch and colleagues,¹²¹ who recently presented the first-in-human PET imaging study with

the PD-L1 antibody ⁸⁹Zr-atezolizumab, a radiotracer able to bind to PD-L1 on the surface of immune and tumour cells (NCT0245398).

Taken together, these data suggest that multiple parameters should be taken into account to identify ideal candidates for immunotherapy in BC. The genomic landscape likely has a role in determining the putative immunogenicity of the tumour¹²²; TIL, PD-L1 expression and immune gene signatures could detect tumours with an inflamed phenotype, which have higher chances of response to ICB.^{111 123} Noteworthy, spontaneous antitumour immune infiltration was shown to be higher in primary tumours with respect to matched metastases (reviewed in Solinas *et al*¹²⁴), suggesting that administration of ICB in the early setting might be more effective than in the advanced setting. Novel immune strategies aiming at inducing (ie, vaccines, adoptive T cell therapy or ICB combinations including CT and RT) or boosting a pre-existing not-sufficient immune response⁴⁴ should be developed in BC to increase the benefit from ICB.

CONCLUSIONS

ICB provides new strategies for the treatment of a variety of neoplastic diseases, including BC, with promising results already demonstrated in the TN subtype in the advanced and neoadjuvant settings. One of the most important remaining challenges is the optimisation of patient selection for ICB, which may require an integrated approach combining multiple predictive biomarkers. The optimal timing of administration, the best combination approach (CT, targeted therapy or RT; administered concomitantly or sequentially) and a standardised evaluation of responses to ICB in clinical trials also represent important research questions to be addressed. Considering the heterogeneous and overall low/intermediate immunogenicity of BC, the best strategy for increasing the efficacy of immunotherapy in this disease is likely the development of multi-modal treatment plans aimed at either boosting or inducing an antitumour immune response.

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