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REVIEW

Clinical development of therapies targeting TGFβ: current knowledge and future perspectives

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Transforming growth factor beta (TGFβ) is a pleiotropic cytokine that plays a key role in both physiologic and pathologic conditions, including cancer. Importantly, TGFβ can exhibit both tumor-suppressive and oncogenic functions. In normal epithelial cells TGFβ acts as an antiproliferative and differentiating factor, whereas in advanced tumors TGFβ can act as an oncogenic factor by creating an immune-suppressive tumor microenvironment, and inducing cancer cell proliferation, angiogenesis, invasion, tumor progression, and metastatic spread. A wealth of preclinical findings have demonstrated that targeting TGFβ is a promising means of exerting antitumor activity. Based on this rationale, several classes of TGFβ inhibitors have been developed and tested in clinical trials, namely, monoclonal, neutralizing, and bifunctional antibodies; antisense oligonucleotides; TGFβ-related vaccines; and receptor kinase inhibitors. It is now >15 years since the first clinical trial testing an anti-TGFβ agent was engaged. Despite the promising preclinical studies, translation of the basic understanding of the TGFβ oncogenic response into the clinical setting has been slow and challenging. Here, we review the conclusions and status of all the completed and ongoing clinical trials that test compounds that inhibit the TGFβ pathway, and discuss the challenges that have arisen during their clinical development. With none of the TGFβ inhibitors evaluated in clinical trials approved for cancer therapy, clinical development for TGFβ blockade therapy is primarily oriented toward TGFβ inhibitor combinations. Immune checkpoint inhibitors are considered candidates, albeit with efficacy anticipated to be restricted to specific populations. In this context, we describe current efforts in the search for biomarkers for selecting the appropriate cancer patients who are likely to benefit from anti-TGFβ therapies. The knowledge accumulated during the last 15 years of clinical research in the context of the TGFβ pathway is crucial to design better, innovative, and more successful trials.

Key words: biomarkers, immunotherapy, TGFβ, tumor microenvironment

INTRODUCTION

Transforming growth factor beta (TGFβ) is a pleiotropic cytokine that plays a key role in embryogenesis and tissue homeostasis. Importantly, the output of the TGFβ signaling depends on the cellular context and the tissue microenvironment, and dysregulation of the TGFβ signaling pathway is involved in several diseases, including cancer.[1–3]

In normal tissues, TGFβ can inhibit proliferation of epithelial cells and promote differentiation, reflecting its tumor-suppressor activity.[4,5] However, in established tumors, TGFβ can play an oncogenic role by promoting cancer cell proliferation, cancer-initiating cell self-renewal, epithelial-to-mesenchymal transition, invasion, tumor progression, metastatic spread, and immune escape.[6–11] In this context, preclinical experimental evidence suggests that TGFβ signaling blockade could exert antitumor activity.[12–14] Furthermore, high levels of TGFβ confers poor prognosis and is associated with early recurrence after surgery, resistance to chemo- or immunotherapy, and shorter survival.[15–18]

In this setting, several potential anti-TGFβ inhibitors are currently under clinical development in phase I/II trials and challenges, such as optimized patient selection and potential combinatory treatments, remain important unsolved clinical questions. Here, we focus on reviewing what has been accomplished on targeting TGFβ as a therapeutic strategy in cancer. We discuss the results of clinical trials and provide an overview on ongoing studies exploring novel

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therapeutic approaches. Finally, we review investigations for potential biomarkers to guide the appropriate selection of cancer patients with the potential to benefit from anti-TGFβ therapies.

THE TGFβ SIGNALING CASCADE IN CANCER

The TGFβ family comprises three isoforms (TGFβ1, TGFβ2, and TGFβ3) that share nearly 70% homology and perform similar biologic activities. TGFβ is secreted by different cell types as a prohormone that is retained in the extracellular matrix. The activation of TGFβ requires proteolytic as well as nonproteolytic processes, which release TGFβ from the extracellular matrix.[19]

Mature TGFβ is a homodimer which forms a complex on the cell membrane with two heterodimers each composed of the TGFβ type II receptor (TβRII) and TGFβ type I receptor (TβRI). After ligand binding, the serine/threonine kinase TβRII phosphorylates TβRI, which in turn initiates the signaling cascade through receptor-activated Smads (Smad2 and Smad3, also known as RSmad; Figure 1).[20] Once phosphorylated, Smad2 and Smad3 are able to form a stable heterotrimeric complex with Smad4, which ultimately translocates into the nucleus, where it regulates the expression of target genes.[21] Smad proteins cooperate with other partners to increase their affinity for DNA; several transcription cofactors have been described.[22–24] As a result, TGFβ-activated signaling can positively or negatively

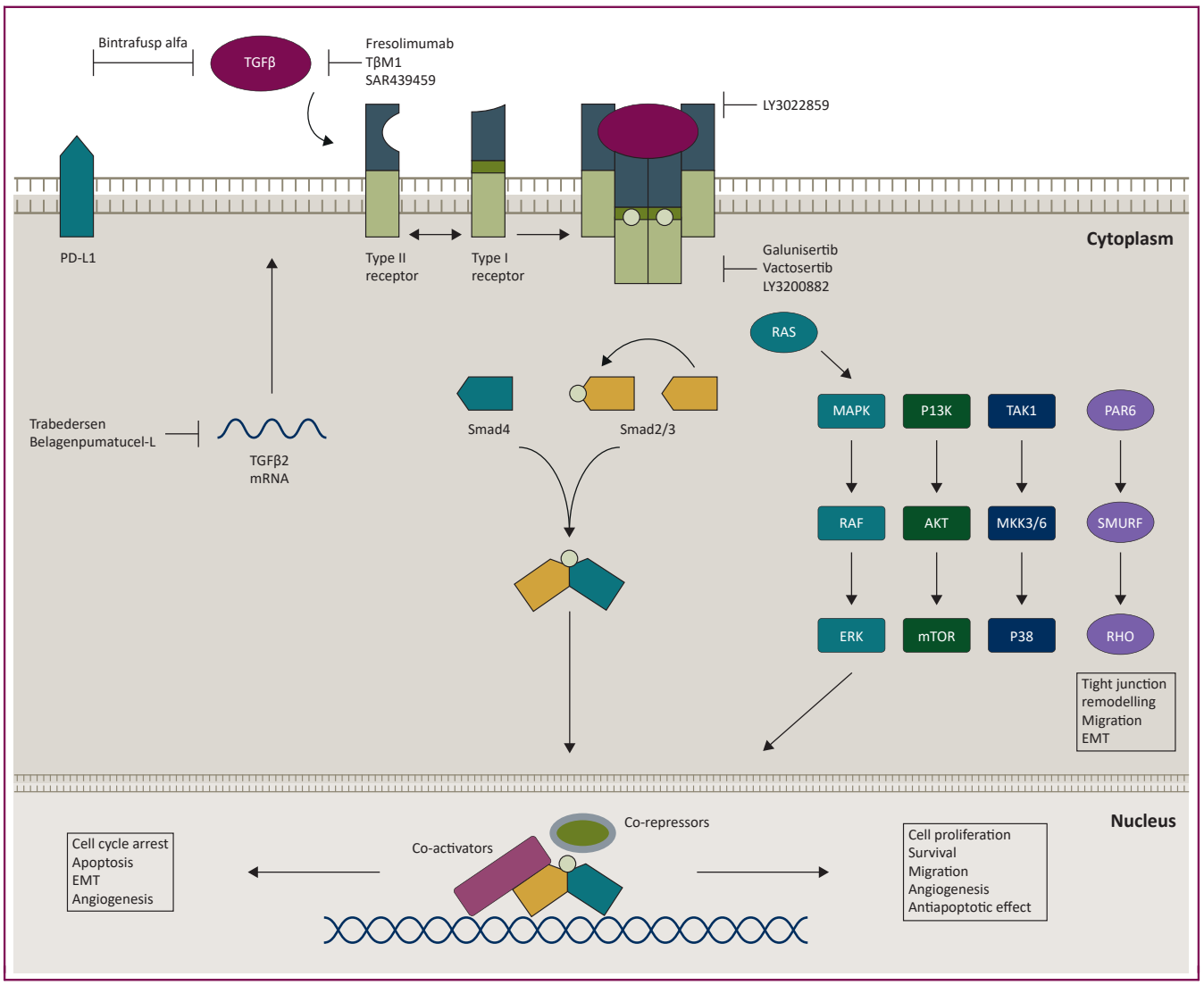

Figure 1. The TGFβ signaling pathway as a therapeutic target.
This pathway represents cell membrane to nucleus signaling. TGFβ forms a heterotrimeric complex with two TβRI and TβRII receptors. Once activated, TβRI phosphorylates Smad2 and Smad3 which is then able to bind Smad4. This tricomplex translocates to the nucleus, and depending on the co-activator/co-inhibitor, regulates the transcription of key genes involved in the cell cycle, survival, and proliferation. In addition, TGFβ can trigger the activation of an Smad-independent signaling pathway, promoting cell survival, proliferation, EMT, and angiogenesis. Different strategies have been developed to inhibit the TGFβ pathway, including TGFβ monoclonal antibodies that sequester the ligand, preventing receptor binding; bispecific antibodies; antisense oligonucleotides; cancer vaccines; and receptor kinase inhibitors. AKT, protein kinase B; EMT, epithelial-to-mesenchymal transition; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; PAR, protease activated receptors; PD-L1, programmed death ligand 1; PI3K, phosphatidylinositol-3-kinase; RAF, rapidly accelerated fibrosarcoma; RHO, RAS homologous protein; SMURF, smad ubiquitin regulatory factor; TβRI, transforming growth factor receptor I; TβRII, transforming growth factor receptor II; TGFβ, transforming growth factor beta.

regulate gene transcription depending on the Smad interaction with other DNA-binding proteins, whose expression can be influenced by different signaling pathways. This explains, at least in part, the plasticity and complexity of TGFβ responses.[1–4] Smad6 and Smad7 are inhibitory molecules that can be induced after TGFβ stimulation, generating negative feedback loop regulation of the TGFβ pathway.[25] Parallel to the Smad signaling cascade, TGFβ can modulate the activation of a plethora of different downstream effectors in a Smad-independent manner, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT), and p38MAPK.[26–33]

TGFβ plays a dual role in cancer. In precancerous conditions, TGFβ exhibits primarily antitumor activity by playing a key role in normal epithelial cells, by means of tissue homeostasis, regulating the cell cycle, apoptosis, and cell differentiation.[34] During tumor progression, the cancer cell escapes the TGFβ antitumor response through the acquisition of mutations in the mediators of the TGFβ pathway both at the receptor and at the signaling level, and also by impairing the antiproliferative and apoptotic response.[35–41] Importantly, in advanced tumors, cancer cells use different strategies to take advantage of the TGFβ signal transforming TGFβ into an oncogenic factor[1–3] (Figure 2).

TGFβ-induced protumor activities rely on its effect on the tumor cell and the tumor microenvironment.[1] It has been shown that TGFβ supports the self-renewal and prevents differentiation of $CD44^{high}/Id1^{high}$ cancer-initiating cells that promote tumor initiation, relapse, and resistance to standard treatments.[42] Moreover, TGFβ can induce cancer-associated fibroblasts and cooperate with vascular endothelial growth factor, hypoxia-inducible factor, platelet-derived growth factor, and other growth factors to modulate angiogenesis.[43–46] TGFβ is one of the most potent immune-suppressive agents and negatively regulates both innate and adaptive immune responses.[47] The multiple mechanisms of immune escape mediated by TGFβ are summarized in Figure 2, and have been reviewed elsewhere.[48]

TRANSLATING TGFβ BLOCKADE INTO ANTICANCER THERAPIES: CLINICAL OUTCOMES AND FUTURE PERSPECTIVES

Based on the body of experimental evidence indicating that TGFβ has the potential to be a good therapeutic target in certain tumors, several anti-TGFβ drugs have been investigated in cancer clinical trials.[43,49] Different strategies have been developed to block TGFβ signaling, including utilizing monoclonal neutralizing antibodies against the TGFβ ligand and its receptor; bifunctional antibodies, such as dual-targeting anti-TGFβ/programmed death ligand 1 (PD-L1) antibodies; antisense oligonucleotides; TGFβ-related vaccines; and receptor kinase inhibitors.[49,50] However, the translation of our knowledge of the basic molecular mechanisms behind the role of TGFβ in cancer into effective clinical outcomes has been relatively slow. The oncogenic and tumor-suppressive role of TGFβ, as well as the complexity of its pleiotropic function in the modulation of cellular and tissue homeostasis, have proven challenging to rapid clinical development of anti-TGFβ agents.[51]

In this regard, TGFβ has a key role in development of cardiovascular organs and in heart remodeling after injury.[52] TGFβRI blockade using two different small receptor kinase inhibitors has been shown to induce heart valve lesions in a rat model.[53] However, the risk of cardiac toxicity is not only limited to the use of small molecule. Recently, it has been reported that treatment with a pan-TGFβ neutralizing monoclonal antibody was associated with an increased risk of bleeding and cardiac toxicity in mice and monkeys.[54]

TGFβ inhibitors are not potent cytotoxic compounds and their antitumor effects depend on the complex interaction between cancer cells, stroma, and the immune system.[48,49] Therefore, the option of combining anti-TGFβ with other anticancer drugs must be considered and the identification of the appropriate therapeutic 'partner' (chemotherapy, radiotherapy, or immune therapy) to combine with TGFβ blockade is likely to be a key factor in improving the antitumor response. A fundamental aspect of anti-TGFβ clinical development is the identification of patients who are more likely to benefit from the blockade of TGFβ signaling. To date, several phase I/II and some phase III clinical trials have investigated the effect of targeting TGFβ in cancer patients (Table 1). Here we summarize current knowledge and discuss future perspectives and scenarios. Table 2 summarizes ongoing studies addressing TGFβ blockade.

Monoclonal antibodies directed against TGFβ ligand or receptors

Several monoclonal antibodies against TGFβ are under development, some isoform specific and others being pan-TGFβ inhibitors. Fresolimumab (GC1008) is a human IgG_4 monoclonal antibody that recognizes all TGFβ isoforms. A phase I study evaluated the safety and the potential clinical activity of fresolimumab in a cohort of 29 patients with previously treated melanoma or renal cell carcinoma.[55] The treatment was well tolerated, and the most frequent drug-related adverse event was reversible cutaneous keratoacanthoma/squamous cell carcinoma and hyperkeratosis. Of note, one patient achieved a partial response (PR) with extensive tumor shrinkage (~90%) that lasted 44 weeks, and six patients had stable disease (SD).[55] In addition, in a small phase II study, 12 patients with relapsed malignant pleural mesothelioma were treated with fresolimumab. Three patients had SD as best response, with median progression-free survival (PFS) of more than 3 months and median overall survival (OS) of 12 months.[56]

TGFβ signaling may represent a mechanism of resistance to radiotherapy.[57,58] In a breast cancer model, irradiation of tumor cells induced the production of TGFβ that promoted DNA damage repair via the ATM (ataxia-telangiectasia mutated) kinase and p53. Radiotherapy can trigger the activation of the immune system, through the induction of immunogenic cell death.[59] Interestingly, preclinical evidence indicates that TGFβ could suppress the immune response induced by radiation, representing a mechanism of immune

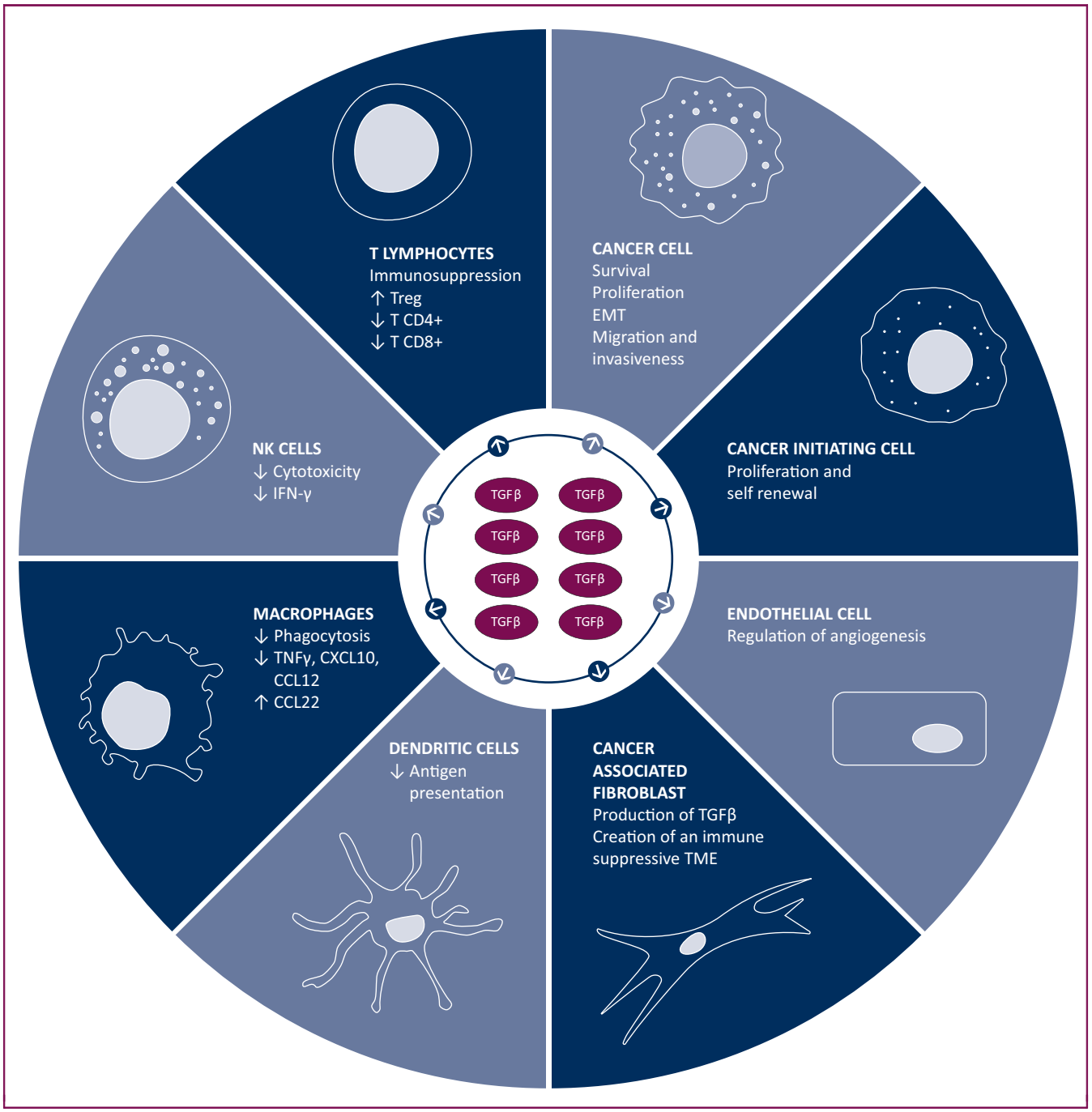

Figure 2. Protumoral activity of TGFβ signaling in cancer.
TGFβ is a pleotropic cytokine and regulates several cellular and tissue functions in a context-dependent manner. The TGFβ pathway is dysregulated in cancer cells, creating an immune-suppressive microenvironment that favors proliferation, tumor growth, angiogenesis, EMT, invasiveness, metastatic spread, and immune escape. EMT, epithelial-to-mesenchymal transition; IFN-γ, interferon gamma; TGFβ, transforming growth factor beta; TME, tumor microenvironment; TNFα, tumor necrosis factor alfa; T_{reg}, regulatory T cell.

escape.[60] A recent phase I trial translated these experiments into the clinic, evaluating the combination of fresolimumab plus radiotherapy in pretreated breast cancer patients with at least three metastatic sites.[61] Toxicity was acceptable with only two of the 23 treated patients developing keratoacanthomas. Although clinical efficacy was very low, with a 13% SD rate (3/23 patients), patients receiving the higher dose of fresolimumab (10 mg/kg) had a significantly higher OS compared with those receiving the lower dose (1 mg/kg fresolimumab; hazard ratio 2.73, 95% confidence interval 1.02−7.30; $P = 0.039$). Moreover, the higher dose correlated with an increase in peripheral blood mononuclear cell counts and a remarkable enhancement in the CD8 central memory pool.[61] A study evaluating the combination of fresolimumab plus stereotaxic radiation therapy in patients with early stage non-small-cell lung cancer (NSCLC) is ongoing (NCT02581787; Table 1). Another phase I clinical trial investigated the monoclonal antibody

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Table 1. Completed clinical trials assessing the safety and efficacy of transforming growth factor (TGF) inhibitors

Study NCT registry number	Agent	Targets	Study population	Number of patients	Phase	Clinical efficacy	Most frequent adverse events
NCT00356460	Fresolimumab	TGFβ1, TGFβ2, and TGFβ3	Advanced melanoma and renal cell carcinoma	29	I	ORR 3, 5% (1 PR) mPFS 2.75 m	Keratoacanthomas Hyperkeratosis
NCT01401062	Fresolimumab RT	TGFβ1, TGFβ2, and TGFβ3	Refractory breast cancer	23	II	ORR 0%	Fatigue Liver enzyme elevations Anemia Keratoacanthomas
NCT01646203	LY3022859	TβRII	Advance solid tumors	14	I	Not reported	Cytokine release syndrome
NCT00557856	PF-03446962	TβRI	Advance solid tumors	44	I	ORR 7% (3 PR)	Thrombocytopenia Fatigue Amylase and lipase elevations
NCT01911273	PF-03446962	TβRI	Refractory hepatocellular carcinoma	24	II	ORR 12%	Thrombocytopenia
NCT01620970	PF-03446962	TβRI	Urothelial cancer	14	II	ORR 0% mPFS 1.8 m mOS 8 m	Thrombocytopenia Fatigue Abdominal pain
NCT01486368	PF-03446962	TβRI	Malignant mesothelioma	15	II	ORR 0% mPFS 1.74 m	Hypertension Fatigue
NCT02116894	PF-03446962 Regorafenib	TβRI Angiogenesis	Pretreated colorectal cancer	11	I	ORR 0% mPFS 1.84 m mOS 4.21 m	Abdominal pain Diarrhea Nausea Fatigue
NCT04296942	Bintrafusp alfa	TβRII and PD-L1	Advanced solid tumors	19	I	ORR 21% (1 CR, 3 PR)	Bullous pemphigoid Lipase increase Colitis Gastroparesis
NCT03427411	Bintrafusp alfa	TβRII and PD-L1	HPV-positive advanced solid tumors	36	I/II	ORR 38.9 % (2 CR, 12 PR)	Colitis Gastroparesis Hypokalemia
NCT02517398	Bintrafusp alfa	TβRII and PD-L1	Pretreated cervical cancer	25	I	ORR 28% (7 PR)	Hypokalemia
NCT02517398	Bintrafusp alfa	TβRII and PD-L1	Refractory head and neck cancer	32	I	ORR 21.9 % (7 PR)	Keratoacanthomas Hyperglycemia Maculopapular rash
NCT02517398	Bintrafusp alfa	TβRII and PD-L1	Pretreated NSCLC	80	II	PD-L1 >1%: ORR 40% PD-L1 >80%: ORR 71%	Pruritus Maculopapular rash Asthenia
NCT02517398	Bintrafusp alfa	TβRII and PD-L1	Pretreated esophageal adenocarcinoma	30	I	ORR 20%	Anemia Cancer pain Gastritis
NCT02699515	Bintrafusp alfa	TβRII and PD-L1	Pretreated gastric cancer	31	I	ORR 22% (2 CR, 5 PR)	Anemia Diarrhea Rash
NCT02699515	Bintrafusp alfa	TβRII and PD-L1	Pretreated biliary tract cancer	30	I	ORR 23% (1 CR, 6 PR)	Interstitial lung disease
NCT02517398	Bintrafusp alfa	TβRII and PD-L1	Refractory colorectal cancer	29	I	ORR 3.4% (1 PR)	Anemia Fatigue Enteritis Blood bilirubin increase
NCT00431561	Trabedersen versus Temozolomide /Lomustine	TGFβ2 RNA Chemotherapy	Recurrent or refractory high-grade glioma	142	IIb	6 months tumor control rate: • Trabedersen 10 µM: 33% • Trabedersen 80 µM: 20% • Chemotherapy: 27%	Nervous disorders
NCT01058785	Belagenpumatucel-L	Cancer vaccine	NSCLC	75	II	Stage III/IV: ORR 15%	Pain Anemia Fatigue
NCT00676507	Belagenpumatucel-L versus placebo	Cancer vaccine	Inoperable or metastatic NSCLC after frontline platinum therapy	532	III	mOS 20 versus 17 m	Allergic reaction Cellulitis
NCT00368082	Adaptive T cell	Immune response	Relapsed Hodgkin lymphoma	8	I	ORR 37.5% (2 CR, 1 PR)	Sepsis
NCT01682187	Galunisertib	TβRI	Advanced solid tumors	65	I	Glioma population: ORR 14%	Thrombocytopenia Thrombosis Dyspnea

Continued

Table 1. Continued

Study NCT registry number	Agent	Targets	Study population	Number of patients	Phase	Clinical efficacy	Most frequent adverse events
NCT01582269	Galunisertib with or without lomustine versus Lomustine	TβRI Chemotherapy	Refractory glioma	180	II	mOS: Galunisertib + Lomustine 6.5 m Galunisertib 8 m Lomustine 7 m	Fatigue Nausea Vomiting
NCT01220271	Temozolomide RT with or without Galunisertib	CT/RT TβRI	Glioblastoma	75	Ib/II	mOS 18.2 versus 17.9 m	Fatigue Nausea Constipation Platelet reduction
NCT01373164	Galunisertib with or without gemcitabine	TβRI Chemotherapy	Inoperable or metastatic pancreatic cancer	170	I/II	mOS 8.9 versus 7.1 m	Neutropenia Platelet count reduction
NCT02154646	Galunisertib + Gemcitabine	TβRI Chemotherapy	Inoperable or metastatic pancreatic cancer	9	I	ORR 0%	Liver enzyme elevations
NCT02734166	Galunisertib + Durvalumab	TβRI PD-L1	Metastatic pancreatic cancer	32	I	ORR 3% mPFS 1.9 m	Liver enzyme elevations Neutropenia
NCT02240433	Galunisertib + Sorafenib	TβRI Angiogenesis	Metastatic hepatocellular carcinoma	14	I	ORR 9%	Hypophosphatemia Hand-foot syndrome
NCT01246986	Galunisertib	TβRI	Metastatic hepatocellular carcinoma	147	II	mPFS 2.7 m part A and 4.2 m part B	Neutropenia Fatigue Anemia

CR, complete response; CT, chemotherapy; CT/RT, chemoradiotherapy; m, months; mOS, median overall survival; mPFS; median progression-free survival; NSCLC, non-small-cell lung cancer; ORR, overall response rate; PD-L1, programmed death ligand 1; PR, partial response; RT, radiotherapy; TβRI, transforming growth factor receptor 1; TβRII, transforming growth factor receptor II.

TβM1, which is directed against TGFβ1, in patients with metastatic solid tumors.[62] Despite an acceptable safety profile, with only one patient experiencing dose-limiting diarrhea, little clinical activity was reported.

The safety and antitumor activity of another pan-TGFβ antibody (SAR439459), as a single agent or in combination with the anti-PD-1 (programmed cell death protein 1) antibody cemiplimab, are being investigated in a trial (NCT03192345) currently recruiting patients with advanced solid tumors (Table 1). LY3022859 is an anti-TβRII IgG_1 monoclonal antibody. Fourteen patients with metastatic tumors were enrolled in a phase I study; however, the trial was prematurely stopped due to the occurrence of uncontrolled cytokine release syndrome, despite prophylaxis with antihistamines and corticosteroids.[63] The antitumor activity of PF-03446962, a fully human IgG_2 monoclonal antibody that blocks TβRI, has been investigated in different tumor types.[64–68] The most frequent grade 3 drug-related adverse events were thrombocytopenia, fatigue, increased serum lipase, and telangiectasia. Interestingly, three patients [one with renal cell carcinoma, one with hepatocellular carcinoma (HCC), and one with NSCLC] experienced a PR as best response, with 12 of 44 (27%) patients achieving SD. Based on the PR obtained in the patient with HCC during dose escalation, an expansion cohort was initiated in these patients.[65] Unfortunately, no PRs were reported, while 12 of the 24 expansion HCC patients (50%) exhibited SD as best response. Median time to progression was 3 months. PF-03446962 antitumor activity as single-agent therapy was also assessed in two phase II studies in urothelial cancer and malignant pleural mesothelioma.[66,67] No major toxicities were reported; however, no signal of clinical efficacy was seen. PF-03446962 was then combined with the small-molecule antiangiogenic multikinase inhibitor regorafenib in patients with chemorefractory metastatic colorectal cancer (CRC)[68]; however, this combination regimen had unacceptable toxicity without any evidence of antitumor activity and the trial was stopped prematurely.

Bifunctional antibodies combining TGFβ and immune checkpoint inhibition

Bintrafusp alfa (M7824) is a first-in-class bifunctional drug, composed of an IgG_1 monoclonal antibody targeting PD-L1 fused with the extracellular domain of two TβRII molecules, which act as a 'trap' sequestering TGFβ in the tumor microenvironment.[69,70] Preclinical evidence has shown that simultaneous blockade of TGFβ and PD-L1 triggers a strong immune response through the combined inhibition of TGFβ-mediated epithelial-to-mesenchymal transition, stimulation of the cytotoxic activity of natural killer cells and $CD8^+$ lymphocytes, and the suppression of Tregs.[70]

In a phase I trial, 19 patients with advanced tumors received bintrafusp alfa.[71] The maximum tolerated dose was not reached with the treatment displaying manageable toxicities. Grade 3 adverse events occurred in four patients: skin infection due to bullous pemphigoid, asymptomatic lipase increase, colitis, and gastroparesis with hypokalemia. Moreover, two patients developed keratoacanthomas that regressed after treatment suspension. Bintrafusp alfa exhibited encouraging clinical activity. One patient with human papillomavirus (HPV)-positive cervical cancer

Table 2. Main ongoing clinical trials evaluating transforming growth factor (TGF) inhibitors

Study NCT registry number	Agent	Targets	Study population	Phase	Recruitment status
NCT02581787	Fresolimumab + SBRT	TGFβ1, TGFβ2, and TGFβ3 RT	Stage Ia/Ib NSCLC	I/II	Active, recruiting
NCT03192345	SAR438459 + Cemiplimab	TGFβ1, TGFβ2, and TGFβ3 PD-L1	Advanced solid tumors	I	Active, recruiting
NCT04349289	Bintrafusp alfa	TGFβRII and PD-L1	Inoperable or metastatic urothelial cancer	I	Active, not yet recruiting
NCT04246489	Bintrafusp alfa	TGFβRII and PD-L1	Platinum-experienced cervical cancer	II	Active, not yet recruiting
NCT04066491	Cisplatin/gemcitabine with or without bintrafusp alfa	Chemotherapy TGFβRII and PD-L1	Metastatic BTC	II/III	Active, recruiting
NCT04220775	Bintrafusp alfa + SBRT	TβRII and PD-L1 + RT	Pretreated SCCHN	I/II	Active, not yet recruiting
NCT03631706	Bintrafusp alfa versus pembrolizumab	TβRII and PD-L1 + PD-1	Untreated advanced NSCLC	III	Active, recruiting
NCT03833661	Bintrafusp alfa	TβRII and PD-L1	Pre-treated BTC	II	Active, recruiting
NCT03524170	Bintrafusp alfa	TβRII and PD-L1	HR+/HER2− pretreated breast cancer	I	Active, recruiting
NCT04296942	Bintrafusp alfa Brachyury-TRICOM Ado-trastuzumab emtansine Entinostat	TβRII and PD-L1 Vaccines HER2 Histone deacetylases	Metastatic breast cancer	I	Active, not yet recruiting
NCT03436563	Bintrafusp alfa	TβRII and PD-L1	Pretreated MSI-H mCRC	I/II	Active, recruiting
NCT03840915	Platinum-based regimen + bintrafusp alfa	Chemotherapy TβRII and PD-L1	Metastatic NSCLC	I/II	Active, recruiting
NCT03840902	Chemo-RT Bintrafusp alfa	Chemo-RT TβRII and PD-L1	Stage III NSCLC	I	Active, recruiting
NCT02452008	Galunisertib + Enzalutamide	TβRI AR	Castration-resistant prostate cancer	II	Active, recruiting
NCT03206177	Carboplatin/ paclitaxel + Galunisertib	Chemotherapy TGFβRI	Ovarian carcinosarcoma	I	Active, recruiting
NCT0266712	Chemo-RT Galunisertib	Chemo-RT TβRI	Locally advanced rectal cancer	II	Active, recruiting
NCT04031872	Capecitabine LY3200882	Chemotherapy TβRI	Pretreated mCRC	I	Active, not yet recruiting

AR, androgen receptor; BTC, biliary tract cancer; MSI-H, microsatellite instable-high; mCRC, metastatic colorectal cancer; NSCLC, non-small-cell lung cancer; PD-L1, programmed death ligand 1; RT, radiotherapy; SBRT, stereotactic body radiotherapy; SCCHN, squamous cell carcinoma of head and neck; TβRI, transforming growth factor receptor I; TβRII, transforming growth factor receptor II.

obtained a complete response (CR), and two patients (one with HPV-positive anal cancer and one with microsatellite instable pancreatic cancer) had prolonged PRs. Furthermore, durable SD occurred in two patients, and a late-onset response was reported after initial disease progression (PD) in a patient with chordoma. Of note, in peripheral blood a nonsignificant increase of B cell and $CD4^+$ T lymphocytes, with a decrease in myeloid-derived suppressor cells, was reported in patients with clinical benefit from bintrafusp alfa.[71] At the AACR 2019 Annual Meeting, pooled data of the dose escalation and expansion cohorts of patients with HPV-positive malignancies including cervical, anal, and squamous cell carcinoma of head and neck (SCCHN) were presented.[72] The overall response rate (ORR) was 38.9% (14/36); two CRs and 12 PRs were observed (one delayed response and three PRs after initial PD). Remarkably, seven patients had durable responses ongoing at the data cut-off. No unexpected toxicities were observed, despite six patients discontinuing treatment due to related adverse events.

Recently, the results of the safety run-in and expansion phases of bintrafusp alfa treatment in cervical cancer were reported.[73] Interestingly, seven of 25 patients experienced a PR, giving a 28% ORR. Biomarker analysis showed that response was irrespective of PD-L1 levels (including PR in one patient with a PD-L1-negative tumor). A single-arm, phase II trial investigating bintrafusp alfa in patients with unresectable or metastatic cervical cancer progressing during or after platinum therapy is ongoing (NCT04246489; Table 2).

Another phase I trial evaluated the activity of bintrafusp alfa in 32 patients with SCCHN, 75% of whom were heavily pretreated.[74] The ORR was 21.9%, however, in the HPV-positive subgroup, and four of eight patients had a PR (50% ORR). These studies highlight the need for further investigations and appropriate tumor tissue analyses to clarify the role of HPV infection as a biomarker of response to anti-TGFβ treatments.

Following these promising clinical results in these three tumor types, the antitumor activity of bintrafusp alfa was

assessed in additional cancer types, including NSCLC and gastrointestinal malignancies.[75–79] In a single-arm phase II study, a cohort of 80 patients with NSCLC received bintrafusp alfa as second-line treatment.[75] An ORR of 40% was seen in the PD-L1-positive population. Moreover, in the patient cohort with high PD-L1 levels (>80% DAKO 73-10 pharmDx kit test), the ORR reached 85%. A global multicenter phase III randomized trial evaluating the efficacy of bintrafusp alfa compared with pembrolizumab as first-line treatment is currently recruiting NSCLC patients with high PD-L1 tumoral expression.[76] At the ESMO 2018 Annual Congress, the results of three phase I clinical trials of bintrafusp alfa in patients with refractory esophageal, gastric, or biliary tract cancer were presented.[77–79] Clinical activity was similar across the three studies with an ORR of approximately 20%. In the biliary tract cancer trial, three fatal adverse events were reported; one patient died due to sepsis and two patients died due to interstitial lung disease.[77] Again, antitumor responses were observed regardless of the PD-L1 levels in the tumor. Collectively, these data suggest that biomarkers other than PD-L1 protein expression by immunohistochemistry (IHC) are needed for optimal selection of patients, at least for patients with non-NSCLC tumors.[78,79]

While immunotherapy has changed the therapeutic paradigm of several malignancies, only a subgroup of patients with microsatellite instable-high CRC respond to immune checkpoint inhibitors.[80] Interestingly, TGFβ is able to activate mechanisms of immune escape in CRC; this provides a strong rationale to combine TGFβ and PD-1/PD-L1 blockade to trigger the immune response in CRC patients.[10] In a phase I trial, 32 patients with metastatic CRC received bintrafusp alfa.[81] Only one PR (maintained for at least 8 months) and one SD were observed. Interestingly, *post hoc* analysis of tumor samples showed that the patient with durable clinical response had a *KRAS* mutant, microsatellite stable tumor with the features of the consensus molecular subtype 4 (CMS4), known to be characterized by activation of the TGFβ pathway.[81,82] Bintrafusp alfa is currently in clinical development in several ongoing trials as monotherapy and in combination with radiotherapy or chemotherapy (Table 1).

Antisense oligonucleotides

Trabedersen is a phosphorothioate antisense oligodeoxynucleotide, which recognizes and binds complementary sequences of TGFβ2 mRNA, preventing protein translation and favoring mRNA degradation.[83] *In vitro* experiments in glioma and pancreatic cancer models have demonstrated that trabedersen reduces the production of TGFβ2, leading to a reduction in cell proliferation, migration, and metastases spread.[83,84] In three phase I/II dose escalation studies in patients with anaplastic astrocytoma and glioblastoma multiforme, trabedersen displayed a good safety profile, without reaching the maximum tolerated dose.[84] Interestingly, some durable responses were reported at doses of 10 and 80 μM. In a phase II randomized study, patients with recurrent anaplastic astrocytoma and glioblastoma multiforme received trabedersen at 10 and 80 μM or standard chemotherapy.[85] The primary end point, which was the 6-month disease control rate, was not reached, and no molecular studies of drug exposure or pharmacodynamic biomarkers were presented. Clinical development of trabedersen in cancer was halted.

Cancer vaccines

Belagenpumatucel-L is a cancer vaccine composed of four irradiated human NSCLC cell lines (which hence lack proliferative potential), transfected with the *TGFβ2* antisense gene. In a phase II trial, NSCLC patients were treated with belagenpumatucel-L at various doses.[86] The treatment had a good safety profile with no serious adverse events. In patients with advanced tumors (stage IIIB and IV) the ORR was 15%. Treatment with higher doses was associated with a better response, with 1- and 2-year survival rates of 64% and 47%, respectively. These preliminary results are promising compared with historical outcomes in the pre-immunotherapy era. Moreover, among patients who obtained a PR, an increase in interferon-gamma, interleukin-6, interleukin-4, and an elevated antibody-mediated response to the vaccine was reported. To boost vaccine efficacy in activating the immune system, a plasmid expressing granulocyte-macrophage colony-stimulating factor was added to belagenpumatucel-L and administered to 23 patients with refractory tumors in a phase I trial.[87] One patient had a long-lasting CR and 21 displayed SD as their best response, with only one patient having PD as best response. No grade 3 or 4 adverse events occurred. To further investigate the efficacy of this approach, a randomized phase III trial of belagenpumatucel-L versus placebo was conducted as maintenance treatment in patients with stage IV NSCLC after platinum therapy.[88] The trial failed to meet its primary end point with no observed difference in OS. Nonetheless, subgroup analysis showed a survival benefit in patients who started treatment within 12 weeks of completing chemotherapy and in those who had received prior radiotherapy. While interesting, the statistical value of this *post hoc* analysis conducted in a small cohort of patients is limited.

Adoptive T-cell transfer

Adoptive T-cell transfer is an innovative and promising treatment for several malignancies.[89] It involves the isolation and reinfusion of T lymphocytes into patients and offers the opportunity for cell engineering to better target cancer cells. Preclinical evidence of antitumor activity via blockade of TGF-β signaling has been demonstrated in a murine prostate cancer model.[90] Engineered T cells with a tumor-reactive T-cell receptor and a dominant-negative TGFβ receptor-II displayed strong antitumor activity. Recently, this approach was tested in the clinic in patients with refractory Epstein–Barr virus–positive Hodgkin lymphoma.[91] In this phase I study, eight patients were treated with DNRII-expressing T cells with specificity for the latent

membrane protein-1 and latent membrane protein-2 Epstein—Barr virus—derived tumor antigens. Of note, two patients had CR (confirmed after 4 years of follow-up), one patient had a PR that lasted 19 months, and four patients achieved SD. Although performing and developing this class of treatment is complex, these findings suggest that blocking the immune evasion induced by TGFβ may be a key for the development of more effective novel immunotherapeutic strategies.

Receptor kinase inhibitors

Galunisertib (LY2157299) is a first-in-class small molecule that targets and binds to the TGFβRI kinase domain. An intermittent regimen with twice-daily administration for 14 days, followed by 14 days off, was evaluated in a phase I trial. No cardiac adverse events occurred and only three patients out of 39 (7.7%) experienced grade 4 toxicities (thrombocytopenia, ischemic stroke, and pulmonary embolism).[92,93] Most patients (30/39) in the dose escalation population had glioma, and the ORR in this glioma population was 16.6% (5/30), including two CRs and three PRs. Moreover, 10 patients (33%) had SD as best response. In a second cohort, 26 patients with refractory glioma received galunisertib plus lomustine. The combination treatment was safe, with no unexpected toxicities. In this cohort, the ORR was 7.7% and SD was reported in four patients. Nonetheless, these two cohorts are not easily compared given the small numbers and major differences in patient characteristics.

This phase I study offered an opportunity for biomarker analyses, with samples available for 21 of 56 patients.[92,93] In this subgroup, five patients displayed an *IDH1/2* mutation. Interestingly, clinical benefit (CR/PR or SD $>$ 6 months) was reported in four of the five (80%) patients with *IDH*-mutated tumors, leading to the launch of a phase II randomized trial. In total, 156 patients with recurrent glioblastoma received galunisertib as single agent or in combination with lomustine (39 and 79 patients, respectively) compared with lomustine monotherapy (40 patients).[94] Unfortunately, no differences in terms of median PFS, OS, and ORR were observed between the experimental treatments and the control arm. For patients with newly diagnosed malignant glioma, the standard of care is surgery followed by concurrent chemoradiotherapy.[95] In a recent phase Ib/II trial evaluating the addition of galunisertib to temozolomide-based radiochemotherapy compared with standard of care, the treatment was well tolerated.[96] However, no difference in median OS were observed and, surprisingly, patients who received galunisertib plus temozolomide-radiochemotherapy had shorter median PFS compared with the standard of care.

In preclinical models of pancreatic cancer, galunisertib enhanced the antitumor activity of gemcitabine,[97] leading to a phase Ib/II randomized study in patients with metastatic pancreatic cancer. The addition of galunisertib to gemcitabine as front-line therapy led to a slight increase in OS versus gemcitabine monotherapy (8.9 versus 7.1 months; hazard ratio 0.79, 95% confidence interval 0.59—1.09).[98] Another small study was carried out in a Japanese population. Seven patients with advanced pancreatic cancer received galunisertib plus gemcitabine.[99] No PRs were observed, and three patients achieved SD. The treatment was well tolerated in both the European and Asian populations. The main limitation of combination regimens in this indication is represented by the chemotherapy backbone. To date, gemcitabine plus nab-paclitaxel or FOLFIRINOX has shown better outcomes compared with gemcitabine alone and they are the preferred first-line options for inoperable or metastatic pancreatic cancer patients.[100] At the 2019 ASCO Annual Meeting, preliminary results were presented of a phase I study of galunisertib combined with the PD-L1 inhibitor durvalumab in recurrent or refractory metastatic pancreatic cancer.[101] The combination was safe; grade 3 hepatic enzyme elevations and grade 3 neutropenia were the most common adverse events, each reported in up to two of the 32 treated patients. One PR and seven SDs were observed, giving a 25% ORR (8/32 patients).

The combination of galunisertib plus the anti-PD-1 nivolumab in advanced solid tumors and refractory NSCLC or HCC has been investigated in a phase I/II study (NCT02423343). The accrual has been completed and results will be presented soon. Furthermore, the clinical activity of galunisertib has been tested in patients with HCC who progressed on or were ineligible to receive sorafenib.[102] Patients were stratified in two cohorts based on alfa-fetoprotein (AFP) levels (AFP $\geq$ 1.5$\times$ upper limit of normal or AFP $<$ 1.5$\times$ upper limit of normal). Interestingly, a survival advantage was reported for patients with high AFP levels compared with the cohort with low AFP (16.8 versus 7.3 months), and furthermore within the high-level cohort, responding patients (reduction in AFP levels of more than 20%) had a greater survival benefit than nonresponders (21.5 versus 6.8 months). Three patients in the low-level AFP group had a PR (3/40, ORR 4.5%).[102] Recently, TGFβ signaling has been suggested as a mechanism of resistance to sorafenib acting via the inhibition of apoptosis. Accordingly, galunisertib enhanced sensitivity to sorafenib in HCC models.[103] The combination strategy was assessed in a small Asian study and found to be feasible. The most frequent adverse events were hypophosphatemia, palmar-plantar erythrodysesthesia syndrome, and thrombocytopenia.[104] In a non-Asian phase II study, 47 patients with HCC received galunisertib (80 or 150 mg b.i.d. for 14 days, every 28 days) and sorafenib.[105] Median time to progression was 4.1 months; OS was 18.8 months. Subgroup analysis showed that responders (TGFβ1 decrease $>$20% from baseline) had longer OS compared with nonresponders (22.8 versus 12 months, $P = 0.038$). PR occurred in two patients, with 21 achieving SD and 13 patients achieving PD. Despite the good tolerability and some initial activity in various tumor types, clinical development of galunisertib was halted in 2017.

Vactosertib (TEW-7197) is a novel potent, highly selective, TGβRI inhibitor.[106] The preliminary results of the phase I study in advanced solid tumors demonstrated that vactosertib was well tolerated and showed signals of clinical activity. Finally, LY3200882, another next-generation TGβRI inhibitor, has been tested in patients with advanced solid tumors.[107] The treatment proved an acceptable safety

profile and demonstrated an antitumor effect. Combinatory strategies of vactosertib and LY3200882 with chemotherapy or immune checkpoint inhibitors are currently under development (Table 1).

POTENTIAL BIOMARKERS TO GUIDE TGFβ THERAPY

Levels of p-Smad2

The clinical developments of TGFβ inhibitors started more than 15 years ago. However, so far, no compound has reached clinical validation and drug approval. In contrast to the excellent preclinical results, TGFβ blockade has displayed disappointing results in most patients and across different tumor types. Although combination strategies with chemotherapy, radiotherapy, immunotherapy, or with other targeted therapies are based on a strong biological rationale, clinical activity of TGFβ inhibitors has only been observed in small subsets of patients. In light of this, identification of tumors that are dependent on the TGFβ signaling pathway is crucial to select patients who could truly benefit from TGFβ-targeted therapies. IHC staining of p-Smad2, a main downstream effector of the TGFβ cascade, and, thus, may indicate the activation of this signaling pathway, is a potential option. Tumor biopsies from a cohort of 25 glioma patients who underwent surgical resection and medical follow-up were analyzed.[108] Based on IHC p-Smad2 staining, tumors were divided into two groups, with 13 samples displaying high p-Smad2 expression [histo-score [H-score] >110] and 12 samples low expression (H-score<110). Interestingly, tumors with high p-Smad2 levels exhibited poor prognosis, with a significantly lower median PFS and OS compared with patients who had low levels. The explanation for this observation was the demonstration that TGFβ increased the proliferation of cancer cells through the induction of platelet-derived growth factor-β.[108] In gastric cancer, p-Smad2 expression levels were increased in tumors with diffuse-type carcinoma, lymph node involvement, and peritoneal metastases and correlated with worse clinical outcome.[109] Similarly, p-Smad2 was reported as a biomarker of aggressiveness in breast and lung cancer.[110,111] Nevertheless, tissue analysis of patients treated with galunisertib suggested that low levels of p-Smad2 were associated with longer response (more than six cycles of treatment).[92,93] However, in this case it should be taken into consideration that the study population was heavily pretreated patients and tissue biopsies were obtained at the time of diagnosis not necessarily reflecting TGFβ activation at the time of starting galunisertib.

Gene expression profiling

Gene expression profiling is another possibility for evaluating TGFβ signaling in the tumor microenvironment. Using a transcriptomic analysis of TGFβ response in four human epithelial cell lines, a TGFβ response signature (TBRS) was developed.[112] The authors applied the TBRS classifier to a transcriptomic analysis of 368 primary breast cancer patients. After 10 years' follow-up, lung and bone metastasis had developed in 39 and 89 patients, respectively. Of note, there was a strong correlation between TBRS+ status and lung recurrence in estrogen receptor-negative tumors. Likewise, another group demonstrated a different TBRS derived from fibroblast rather than epithelial cells, predicted relapse in CRC independent of the patients' clinical characteristics, and was associated with poor prognosis.[12] Similarly, a transcriptomic analysis of a cohort of patients with urothelial cancer revealed that a TGFβ gene signature in cancer-associated fibroblast was associated with unresponsiveness to the immune checkpoint inhibitor atezolizumab.[113] Moreover, a TGFβ signature obtained in mice hepatocytes was associated with a more aggressive phenotype, with increased risk of recurrence and with shortened survival in HCC.[114] Finally, a TGFβ signature derived from the chronic treatment of MCF10A epithelial breast cancer cell lines was able to predict the activation of TGFβ signaling.[115] Interestingly, SCCHN HPV-positive tumors with loss of TGFβ function displayed an impairment in DNA repair and increased sensibility to cisplatin and radiotherapy.[115] These data suggest that gene expression profiling could be a useful tool to identify tumors that activate TGFβ signaling, and thus for selecting patients to be included in future clinical trials.

The MoTriColor project is an international consortium, in which patients with metastatic CRC receive personalized treatment, based on tumor molecular profiling. In one of the treatment cohorts, patients with a TGFβ-like signature receive the TGβRI inhibitor LY3200882 in combination with capecitabine as second-line treatment (NCT04031872).

Blood and tissue biomarkers

Analysis of TGFβ1 levels in blood and tumor samples of patients treated in clinical trials could represent a useful strategy to understand possible biomarkers of response or resistance to TGFβ blockade. In the HCC trial evaluating the combination of galunisertib and sorafenib, reduction in TGFβ1 plasmatic levels after treatment correlated with longer OS, although there was no apparent association with increased tumor shrinkage.[105] Recently, a biomarker analysis of pancreatic cancer patients treated with galunisertib and gemcitabine versus gemcitabine was reported.[116] In this study, patients who displayed a steeper decrease in TGFβ1 showed improved OS for the combination treatment compared with standard of care (12.7 versus 9.7 months, $P = 0.1892$). The authors analyzed several circulating markers. Three microRNAs were associated with reduced prognosis (miR-21-5p, miR-210-3p, and miR-148b-3p), whereas two of them correlated with better OS in the experimental arm (miR-424-5p and miR-10b-5p).

In the small cohort of metastatic CRC patients treated with bintrafusp alfa, only one PR was observed in a patient with a microsatellite stable, CMS4 tumor.[81] CMS4 CRC has a mesenchymal phenotype and may be characterized by upregulation of TGFβ signaling.[82] However, the CMS4 gene signature does not necessary predict TGFβ pathway activation but rather a mesenchymal phenotype, and therefore

does not represent an ideal biomarker for selecting patients with a TGFβ-dependent cancer.

Immune biomarkers

In the current era of immune therapy with immune checkpoint inhibitors, the evaluation of PD-1/PD-L1 is one of the most used biomarkers of response. However, there are several controversial issues regarding the different diagnostic tests and the role of PD-1/PD-L1 expression.[117] With the exception of NSCLC, PD-L1 levels have not correlated with clinical benefit in any of the patient cohorts treated with bintrafusp alfa.[71–99,102] By contrast, the NSCLC cohort of patients with high PD-L1 expression had a very high extent of tumor regression with bintrafusp alfa (more than 80%).[75] It has been established that tumors with PD-L1 in >50% of the cells are more likely to benefit from pembrolizumab.[118] Thus, despite the strong rationale for combination treatment, it is very difficult to assess the real contribution of the addition of TGFβ blockade with bintrafusp alfa. The expected results of the phase II randomized trial of bintrafusp alfa versus pembrolizumab in this NSCLC population will shed light on this unresolved question.[76]

CONCLUSIONS

TGFβ is a pleiotropic cytokine that, depending on the cellular and tissue context, can activate either protumor or antitumor responses. Cancer cells are able to escape the antiproliferative TGFβ response and take advantage of the TGFβ protumor functions. Despite extensive preclinical and translational research showing evidence that blocking TGFβ is a potentially effective therapeutic strategy, the translation from bench to bedside has been slow and to date not highly successful. None of the TGFβ inhibitors that have been evaluated in clinical trials are currently approved for cancer therapy. The fact that TGFβ inhibition does not lead to direct cytotoxic activity implies that it is challenging to observe clinically meaningful tumor regression with single-agent inhibitors, and combinatory approaches should be considered. The choice of the right therapeutic partner is a fundamental aspect to address. In the era of immunotherapy, the combination of TGFβ blockade with immune checkpoint inhibitors represents an appealing strategy. However, even in this case only a subset of patients will respond to treatment. This highlights the necessity to go back to the laboratory from the clinic and perform a thorough analysis of the characteristics of patients treated with TGFβ inhibitors (including analyses of blood and tumor molecular characteristics), to identify potential biomarkers of response. In this scenario, the use of gene expression profiling rather than a single biomarker could facilitate the selection of cancer patients who might benefit from TGFβ blockade. After more than 15 years of work, there is light at the end of the tunnel and TGFβ is now considered an appealing therapeutic target meriting further translational investigation in the context of well-designed clinical trials.

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