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INHIBITION OF TGFβ SIGNALING AND ITS IMPLICATIONS IN ANTICANCER TREATMENTS

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The transforming growth factor-β (TGFβ) is a potent regulator of tumorigenesis. In cancer, two distinctive behaviors of TGFβ have been reported as a tumor suppressor at early stage of the disease, and as a tumor promoter at later stages. The past decades, the dualistic role of TGFβ has garnered a lot of attention. As a result, cancer researchers' has been tasked to elucidate how TGFβ signaling may lead to metastatic dissemination, how to tackle carcinogenesis and which therapeutic strategies should be adopted. Consequently, TGFβ signaling pathways have been considered as appropriate targets for cancer therapy. The TGFβ therapeutic strategies have emerged at three levels: ligand, ligand-receptor interaction and intracellular signaling level. Promising inhibitors of TGFβ signaling have entered clinical trials and shown encouraging results. Here we review the three strategies of TGFβ signaling inhibition and theirs applications in treatment of cancer.
***Key Words*: TGFβ, cancer, inhibition of TGFβ signaling, therapeutic strategy.**

The TGFβ is a potent cytokine endowed with remarkable functionalities allowing it to perform multiple tasks. Among these tasks, there are regulation of cell proliferation, differentiation, and apoptosis. The cell type and the cell environment may influence the function of the cytokine, enabling it to control multitudinous processes either normal or pathological. As examples of physiological events related to TGFβ signaling are embryogenesis, wound healing and tissue homeostasis. Regarding pathological disorders, such as cancer, arteriosclerosis, fibrosis and Marfan's syndrome, compiling evidences have shown that loss of control of the TGFβ signaling is associated with these conditions. In the neoplastic transformation, TGFβ plays two conflicting roles of a tumor suppressor and a tumor promoter. The inhibition of TGFβ signaling pathways may be achieved at three levels in the TGFβ signaling pathways. The first strategy is to target the TGFβ ligand. The second strategy is to affect the interaction between the TGFβ ligand and the TGFβ receptors. Finally, the third strategy focuses on the receptor-mediated signaling cascade. There is a considerable diversity of inhibitors designed to approach each of the three levels. These inhibitors have already shown different beneficial aspects in preclinical and clinical studies.

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Abbreviations used: Abs – antibodies; ALK5 see TβR-I; ASO – antisense oligonucleotides; EMT – epithelial-to-mesenchymal transition; mAbs – monoclonal antibodies; miRNA – micro interfering RNA; RNAi – RNA interference; R-Smads – receptor-regulated Smads; SARA – Smad anchor for receptor activation anchorage; siRNA – short interfering RNA; Smad – small mothers against decapentaplegic; TGFβ – transforming growth factor beta; TβR-I – type I transforming growth factor receptor; TβR-II – type II transforming growth factor receptor; TβR-III – type III transforming growth factor receptor; TβRs – TGFβ receptors; T-cells – T lymphocytes; TF – transcription factors.

TGFβ: STRANGE CASE OF "TGFβ" DR JEKYLL OR MR HYDE?

Carried out by Robert W. Holley in the early 70s, the initial study leading to the discovery of TGFβ and its naming as a *transforming* growth factor were based on its ability to induce malignant behavior of normal fibroblasts. This brought the idea that TGFβ might be a key factor in transformation of cells [1, 2]. Meanwhile, other experiments indicated a conflicting function of TGFβ on cells, that of a tumor suppressor [3]. Today, it is well established that conceded roles to the cytokine are cancer stage dependent [3, 4]. In the early stage of cancer development, TGFβ can suppress tumor growth, whereas in the late-stage it can take on role of a tumor promoter, favoring spreading of metastasis [5]. TGFβ is assumed to arbitrate a broad range of physiological processes e.g. wound healing, proliferation, epithelial homeostasis, embryogenesis and apoptosis but also pathological processes such as Marfan's syndrome, fibrosis, carcinogenesis including angiogenesis and epithelial-to-mesenchymal transition (EMT) [6–8]. Even three decades after its discovery, it is a difficult task to ascribe one single role to the TGFβ in the case of carcinogenesis. The "whimsical" behavior of this cytokine leads to the conclusion that it might be both, a kind of "Guardian angel" by its ability to inhibit tumor proliferation, but nevertheless a kind of "Devil" by its aptitude to enhance metastasis spreading, and for that reason, would deserve the dual title of "Dr Jekyll/Mr Hyde" [9, 10].

TGFβ SIGNALING: "TO SMAD, OR NOT TO SMAD?" [11]

The following model for TGFβ signaling pathway trough Smad proteins has been suggested in several reports (Fig. 1) [12–17].

The signal is triggered through binding of the mature TGFβ ligand to the extracellular domain of type II TGFβ receptor (TβR-II) or to the accessory receptor, type III TGFβ receptor (TβR-III) which transfers TGFβ to TβR-II. Following the transfer of the cytokine to TβR-

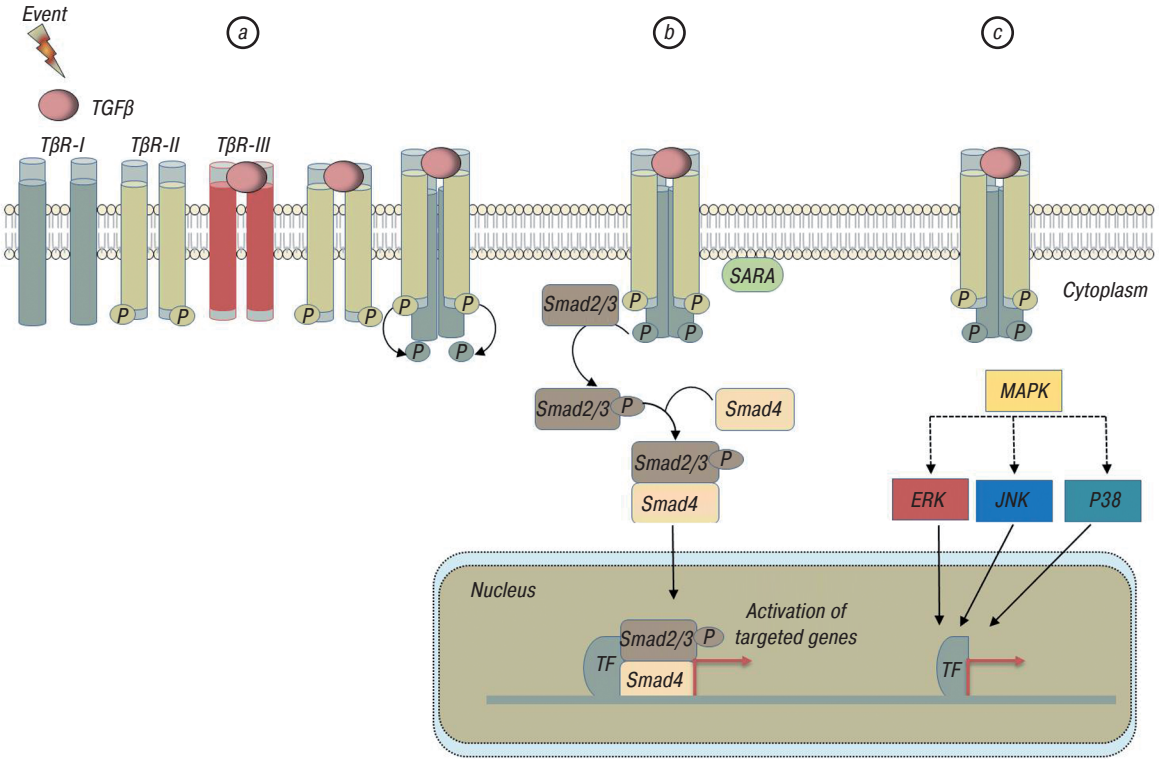

Fig. 1. TGFβ Smad and non-Smad signaling pathways from initiation to nucleus. (*a*) Signal initiation. After the secretion of TGFβ, the active cytokine can bind TβR-II or the accessory receptor TβR-III which presents it to TβR-II. TGFβ binding to TβR-II leads to bridging of TβR-I into the complex, and allows TβR-II to phosphorylate TβR-I. (*b*) Smad-dependent pathway. The R-Smads (Smad2 and Smad3) are activated by TβR-I. However their recruitment can be eased by auxiliary proteins, e.g. SARA. These activated R-Smads complex a Co-Smad (Smad4). Finally, the complex is imported into the nucleus, where with the help of other co-operators it regulates expression of targeted genes. (*c*) Smad-independent pathway. TGFβ can regulate expression of a wide range of genes by inducing other signaling cascade independently of the Smad-dependent pathway, such as shown MAPK

II, the constitutively active receptor TβR-II recruits and phosphorylates the signaling type I TGFβ receptor (TβR-I) [18]. The TβR-I acts downstream of the type II, and determines the specificity of intracellular signals by phosphorylating a subset of transcriptional cytoplasmic factors (TF), major linchpin of the signaling pathway Smad2 and Smad3. Also called R-Smads (receptor-regulated or activated Smads), for the reason that Smad2/3 protein activation is under the control of the receptor TβR-I [13]. Several phosphoisoforms of the R-Smads have been identified as a result of TGFβ and Ras/MAPK pathways activation [19, 20]. The phosphorylation of these first intracellular mediators stimulates their interactions with Smad4 (Co-Smad), a co-mediator and other protein partners e.g. SARA (Smad anchor for receptor activation anchorage), Dab2, Endofin, Axin, etc. [10, 21]. Once assembled, the complex Smad4-Smad2/3 shuttles to the nucleus where it may exert distinct transcriptional control [22, 23]. Besides this Smad-dependent pathway which is doubtless the most-well known TGFβ signaling pathway, other pathways have been identified, clustered and termed as Smad-independent pathways [24, 25]. These other signaling cascades can be activated by TGFβ, and in such a way can orchestrate the transcription of target genes. Among them are described various branches of the MAPK, Rho-like GTPase and PI3K/AKT pathways [24–28]. It is broadly accepted that during carcinogenesis, the Smad-dependent pathway correlates with the anti-proliferative or tumor suppressor functions of TGFβ, and that the Smad-independent pathways are involved in TGFβ pro-malignant functions [29].

THREE APPROACHES TO INHIBIT TGFβ SIGNALING

Taking account of TGFβ involvement in carcinogenesis (tumor suppression and tumor promotion), the targeting of TGFβ signaling pathway for therapeutics purposes was an ineluctable choice. By dint of intensive works, over fifteen years, the therapeutic strategies to disrupt TGFβ signaling have emerged at three levels: ligand, receptor-ligand interaction and intracellular signal transduction (Fig. 2). Several inhibitors have entered clinical trials, from phase I to III. The Table 1 summarized the current knowledge on therapeutic strategies to impede TGFβ signaling.

Intervention on the ligand level

The signaling pathway's first component is the ligand. Therefore an interest in targeting the transcriptional products of TGFβ-coding genes in order to restrain the synthesis of TGFβ has been applied. The technique called Gene Silencing by RNA Interference (RNAi) allows regulation of the gene expression. The sRNAi technology is based on two types of small molecules of RNA: the micro interfering RNA (miRNA) and the short interfering RNA (siRNA) [30]. These small molecules act by binding complementary sequences on specific mRNAs, therefore preventing translation and in that way silencing TGFβ genes. Binding of an-

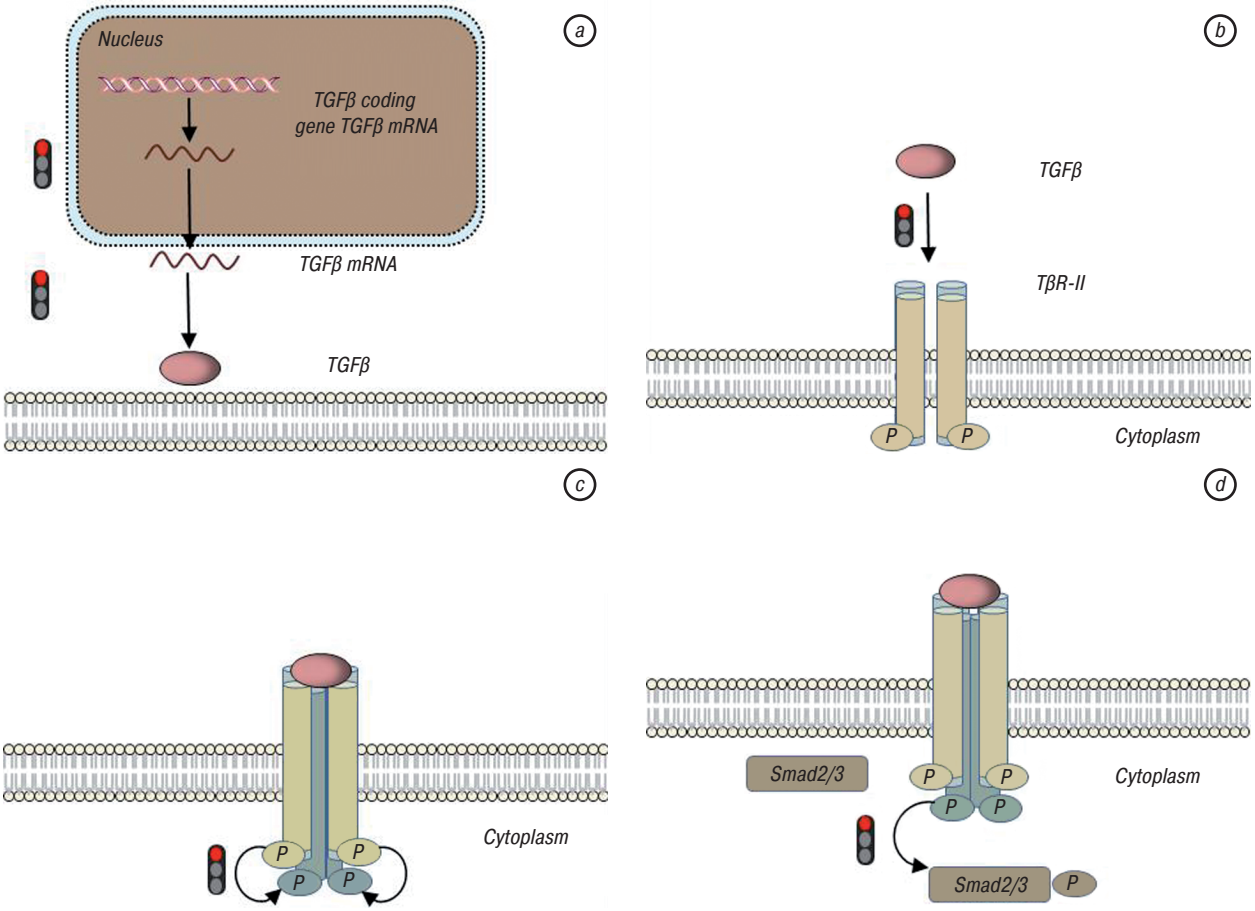

Fig. 2. Current strategies to impede TGFβ signaling. (*a*) Direct or indirect inhibition of TGFβ secretion. The first measure consists to prevent the secretion of TGFβ directly by action on transcript products using mAbs or ASOs or via indirect routes by decreasing the secretion rate of the cytokine. (*b*) Inhibition of TGFβ-receptor binding. Another therapeutic line lies in the neutralization of the secreted cytokine by mAbs, soluble receptors or natural TGFβ inhibitors, and therefore blocking the ligand-receptor binding. (*c*) Inhibition of TGFβ receptor activation. Antagonism of the transduction signal through hampering of TβR-I may be achieved by inhibitors interfering with the ATP-binding pocket or the Smad-binding pocket of the kinase. (*d*) Inhibition of Smad activation. Finally to stymie the progression of TGFβ signaling on the transduction level one may target Smad2/3 directly. Strategies (*a*), (*b*) and (*c*) are currently being explored, and discussed in the text

tisense oligonucleotides (ASO) to RNAs and targeting TGFβ mRNA made silencing of TGFβ gene possible [31, 32]. The trabedersen, also termed AP-12009, from Antisense Pharma is an ASO responsible for silencing of TGFβ2 gene [29]. In recurrent and refractory high-grade glioma patients, promising results have already been obtained and led to the clinical trials phase III [33, 34]. However, in spite of a high specificity, trabedersen administration remains an issue. In high grade glioma patients a neurosurgical intervention is required to set-up a relatively complicated drug delivery system. This drug delivery system includes a pump placed outside the body which is connected to an internal catheter flowing to the brain [35–37]. Currently, Antisense Pharma is performing phases I/II clinical trials in pancreatic neoplasms, melanoma and colorectal neoplasms using intravenous delivery that already show encouraging efficacy [38]. By neutralizing the TGFβ2 mRNA produced by tumor cells, the Belagenpumatucel-L or Lucanix™ from NovaRx was expected to restore tumor antigen recognition by immune effector cells i.e. T-cells [39]. In patients with non-small cell lung cancer, results from a phase II study suggest that the number of circulating tumor cells at baseline appears to correlate with the overall survival. Such results highlight that further explorations remain needed [40, 41]. Although, the published reports are not enough clear regarding the adverse effects, this lack of information may have a considerable impact on the future safety status of neutralizing RNAs as drugs [36].

Intervention on the ligand-receptor interaction level

Drugs of the second level target interaction between the ligand and the specific receptor. To date intervention on the ligand-receptor level encompasses three categories of compounds: monoclonal antibodies (mAbs), natural TGFβ inhibitors and soluble TGFβ receptors (fusion constructs). Here we focus on the mAbs due to the broad use in clinic of antibodies as drugs targeting different signaling receptors. The application of mAbs as a therapeutic end (i.e. immunotherapy) for cancer can be explained by their high specificity [42–44]. Several investigators have demonstrated in cancer mice models that a neutralization of the three isoforms of TGFβ circulating in the bloodstream using several mAbs e.g. 1D11 and 2G7 affected the tumor growth [6]. The mAbs list

Table. Overview of therapeutic TGFβ signaling inhibitors used in pre-clinical and clinical studies. (A) Direct or indirect inhibition of TGFβ secretion. (B) Inhibition of TGFβ/receptor binding. (C) Inhibition of TGFβ receptor activation. (D) Inhibition of Smad activation

	Target	Generic name	Status	Application	References
(A)	TGFβ mRNA	AP-15012	discovery	oncology	29
	TGFβl mRNA	AP-11014	adv. preclinical	oncology	58
	TGFβ2 mRNA	AP-12009 (Trabedersen)	III recruiting	oncology	33, 34, NCT00761280
	TGFβ secretion	Tranilast	preclinical	various	59, 60–62
	TGFβ2 secretion	Bevacizumab® (Avastin)	II recruiting	various	63, NCT00121134 NCT00733408
	TGFβ2	Glionix™	II–III initiated	oncology	64, 65
	TGFβ2 secretion	Lucanix® (Belagenpumatucel-L)	III recruiting	oncology	40, 41, NCT00676507
(B)	TGFβI	Metelimumab® (CAT-192)	II discontinued	scleroderma	47, 49
	TGFβ2/3	Lerdelimumab® (CAT-152/Trabio)	III discontinued	various	46, 49, 66
	Pan TGFβ	Fresolimumab® (GC-1008)	I	various	45/NCT01284322 NCTO1401062
	Pan TGFβ	SR-2F	preclinical	oncology	67
	Pan TGFβ	1D11	preclinical	oncology	36
	Pan TGFβ	2G7	preclinical	oncology	68–70
(C)	TβR-I	A-83-01	preclinical	oncology	71
	TβR-I	GW6604	preclinical	fibrosis	72, 73
	TβR-I	IN-1130	preclinical	fibrosis	74
	TβR-I	Ki26894	preclinical	oncology	75
	TβR-I	LY2157299	I/II	oncology	76, NCT01220271 NCT01246986 NCT01373164
	TβR-I	LY364947 (HTS-466284)	preclinical	various	36, 77
	TβR-I	LY550410	preclinical	various	48
	TβR-I	L Y5 73636-sodium (Tasisulam)	I/II/III suspended	oncology	78, 79
	TβR-I	LY580276	preclinical	various	48
	TβR-I	NPC-30345	preclinical	various	80, 81
	TβR-I	SB-431542	preclinical	oncology	51, 52
	TβR-I	SB-505124	preclinical	various	53
	TβR-I	SD-093	preclinical	oncology	81, 82
	TβR-I	SD-208	preclinical	various	82, 83
	TβR-I	Sml6	preclinical	oncology	84
	TβR-I	SM305	preclinical	fibrosis	85
	TβR-I	SX-007	preclinical	oncology	86
	TβR-I	Antp-Sm2A	preclinical	oncology	57
	TβR-I /II	LY2109761	preclinical	oncology	87
		P144 (Disitertide)	preclinical/II	various	88, 89
		P17	preclinical	various	88, 90
	TβR-III	sRIII	preclinical	oncology	91, 92
(D)	Smads	Trx-CBP	preclinical discontinued	oncology	93
	Smads	Trx-FoxHlb	preclinical	oncology	93
	Smads	Trx-Lefl	preclinical	oncology	93
	Smad2/3	Trx-SARA	preclinical	oncology	94
	Smad3	SiS3	preclinical	fibrosis	95

Abbreviations: adv. – advanced, NCT – Clinical Trial Registry Numbers. Source: ClinicalTrials.gov

targeting TGFβ includes GC1008, CAT-152 and CAT-192 [45–47]. All three Abs are up to date the most developed antibodies in clinical trials [29, 36, 48]. The pilot study and the phase II studies carried out on patients with advanced malignant melanoma or renal cell carcinoma have shown a reasonable tolerance vis-à-vis GC1008 and a neutralization of TGFβ, holding promise of a novel cancer therapeutic agent [45]. However several adverse effects have been noticed, such as fatigue, headache, epistaxis, gingival bleeding, skin rash [6, 45]. Nonetheless, a phase II protocol expansion study is recruiting patients with metastatic malignant melanoma. This protocol allows monitoring of the GC1008 effects in blood samples from patients. A phase II study of patients with breast cancer is planned with GC1008 [6]. The CAT-192 or Metelimumab® and the CAT-152 or Lerdelimumab® are human IgG4 mAbs directed against TGFβ1 and TGFβ2, respectively. Early clinical studies have suggested that Metelimumab® was safe and well tolerated, with a long half-life and has completed phase I/II studies with patients with cutaneous systemic sclerosis. No trials in cancer have yet been initiated [49]. It is important to bear in mind that the antibodies must surpass substantial obstacles to reach the tumor mass [36, 50]. Among them, the physical barrier including vascular endothelium, stromal barriers, high interstitial pressure and epithelial barriers may explain in part why the therapeutic antibodies have a moderated success in treatment of cancer as compared to fibrotic disorders [36, 44, 50].

Intervention on the intracellular signaling level

The transforming factor receptors (TβRs) are the gateways of the intracellular signaling. Therefore, drugs blocking TGFβ receptors intracellular activity have been developed, thus constituting the third group of inhibitors. Most of them are small molecule inhibitors targeting the kinase of TβRs. However, others inhibitors target Smads interaction with TβR, using peptide aptamers to Smad (Table 1). This section of our report focuses on the inhibitors targeting the TβRs. Among these inhibitors there are two categories; the small and the large molecules. The currently developed inhibitors may have an imidazole scaffold, such as SB-431542 and SB-505124, or a pyrazole scaffold such as LY-580276 [36, 48]. Most of these inhibitors are

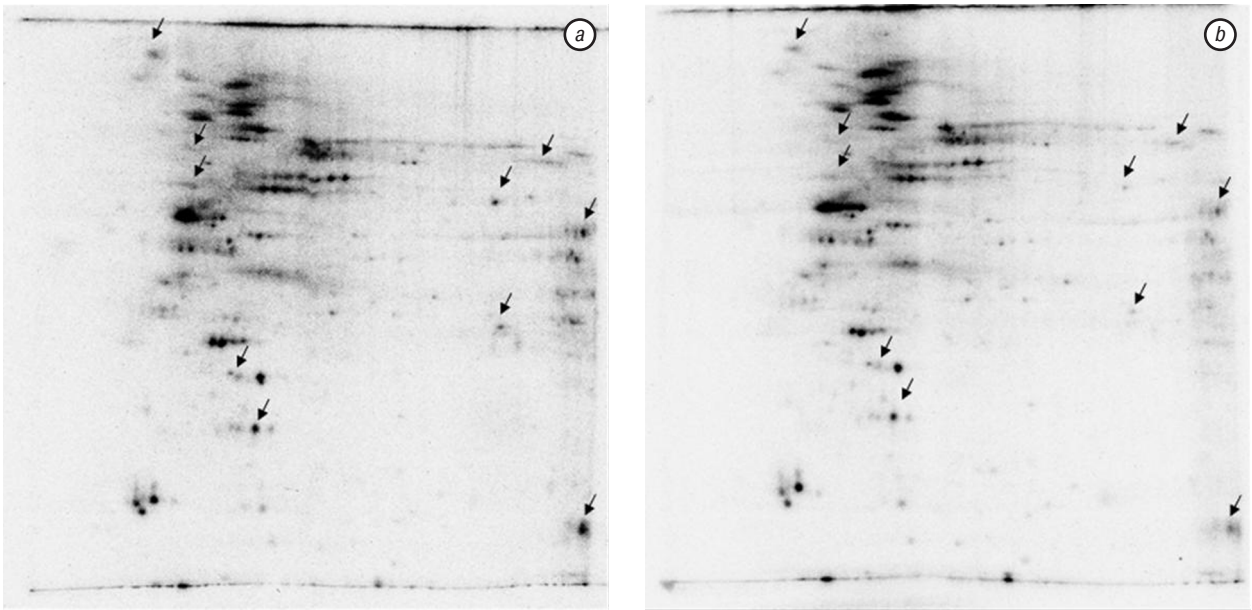

Fig. 3. Screen for specificity of a kinase inhibitor. *a* — without inhibitor; *b* — +SB-431542. ATP-binding site interfering inhibitor SB-431542 was added to cell extract (right 2D gel) or not (left 2D gel), *in vitro* kinase reaction was performed under conditions promoting autophosphorylation of kinases. Proteins were separated, and ^{32}P incorporation was detected after exposure in a PhosphorImager™. Arrows show migration positions of proteins which were affected by addition of SB-431542

directed towards TβR-I kinase catalytic ATP-binding site [48]. Two representative candidates are being developed by GlaxoSmithKline, e.g. SB-431542 and SB-505124. Both aim to fully abrogate or strongly down-regulate the TβR-I mediated signaling cascade. These imidazole-based compounds have already shown remarkable effects at nanomolar concentrations, comprising inhibition of the TGFβ-induced Smad phosphorylation, as well as inhibition of a reporter gene [51–53]. Finally, the TβR-I blockade has shown effects on cellular responses such as the cell cycle arrest and EMT of mammary epithelial cells *in vitro* [6, 51]. In contrast to its analog (i.e. SB-431542), SB-505124 has been revealed to be three to five times more potent [53]. However it has been shown that these inhibitors may not be specific to TβR-I. Fig. 3 shows a proteomics screen to evaluate the specificity of SB-431542. This screen detected a number of phosphorylated proteins which were affected by the drug. The assay was designed to detect predominantly auto-phosphorylated kinases. Detection of multiple targets of the drug underscores importance of the unbiased evaluation specificity of the drug. The lack of high specificity could be explained by the inherent analogous structure shared by several kinases on their ATP-binding pocket, e.g. p38, bringing Lahn *et al* to voice the concern that such off-target inhibition might be liable to unexpected toxicity [36]. Finally, even if such molecules present the particularly advantages of an oral administration and a high selectivity, they remain nonetheless not enough specific and should be carefully monitored in future clinical trials [54–56]. This is in view of certain cases of resistance to the drug, or side-effects such as cardiac conditions reported in the literature [55, 56].

Whilst some researchers have focused in recent years to create inhibitors targeting the ATP-binding site, another approach to target the kinase on the substrate-biding site has been reported [57]. This novel strategy aims to inhibit signaling by blocking the substrate-binding site of the TβR-I kinase with peptides mimicking the Smad2. This new class of inhibitors acts as "decoys" which once occupying the Smad2-binding pocket, prevent Smad2 phosphorylation, and hence its activation. This idea should by definition allow a high specificity that some ATP-mimicking inhibitors do not offer. So far, only one group has produced and investigated the effects of such compounds. The results have shown that this kind of compounds can indeed disturb TGFβ signaling by blockade of TβR-I *in vivo* and *in vitro*, in Mv1Lu cells. On top of this, there have been shown that those new inhibitors affected TGFβ1-dependent phosphorylation of endogenous Smad2, as well as gene stimulation. Finally, these pseudo-substrates have shown higher efficiency vis-à-vis the TβR-I kinase than to kinases of other type I receptor of TGFβ and BMP family [57]. Nowadays, investigations on normal and cancer celllines are ongoing. In view of the encouraging results, it is clear that development of pseudo-substrate inhibitors may lead to new therapeutic strategies to impede TGFβ signaling.

CONCLUSION

As metastasis dissemination remains the major cause leading to death of cancer patients, significant efforts have been undertaken over the years to tackle cancer by blockade or at least by decreasing development of the metastasis. To face this challenge, the inhibition of TGFβ signaling appears as a therapeutic strategy. This strategy has been approached at three levels: ligand, receptor-ligand binding and intracellular signaling levels. Among them, the ASOs and the mAbs technologies are the most advanced. Yet, the inhibitors likely to experience a fast growth will undoubtedly be the small-molecules drugs. Novel type of inhibi-

tors, e.g. substrate-mimicking drugs, requires further developmental efforts. However, already now we have examples of successful application of inhibition of TGFβ signaling for the benefit of cancer patients. This ensures that TGFβ inhibitors came into anticancer treatment to stay. The inhibitors have been tested in a number of assays, and have shown their efficiency. However, developmental work requires much more. Taking into account potential benefit for patients and results of clinical trials with other types of TGFβ signaling inhibitors, there is a strong support to continue development of the substrate-mimicking inhibitors.

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