A Cautious Note on Thalidomide Usage in Cancer Treatment: Genetic Profiling of the TBX2 Sub-Family Gene Expression is Required

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ABSTRACT
Thalidomide is still by excellence the mysterious drug that fascinated, blurred, misled, and changed the scientific community perspectives and policies. It was introduced in the 1950’s as a sedative drug, then shortly withdrawn because of the devastating birth defects that affected tens of thousands throughout more than 40 countries. Back into the market in the mid-1990’s and 2000’s the drug is now being used to treat skin immune-related conditions and some cancers like multiple myeloma. Despite numerous beneficial effects which led to the development of new analogs, its direct mechanisms of action are still elusive. The identification of CRBN and TBX5 as potential direct ligands for this drug have opened the way to better understand its efficiency and its failure.

We hereby review these mechanisms and provide evidence that could explain why thalidomide failed to make it as a drug of choice in lung cancer treatment. Linking the genetic signature of TBX2 subfamily in these tumors to their inability to respond properly to thalidomide raises concerns of worsening lung cancer patients’ health if this drug is utilized.

Introduction
Thalidomide, or N-phthalimidoglututarimide [C13 O4 N2 H9], a sedative drug (Contergan®) that was introduced back in the mid-1950’s for treating morning sickness in pregnant women [1]. Few years later, it was removed from the market due to its devastating teratogenic effect on children whose mothers used this drug during their pregnancies. These newborns suffered mainly from phocomelia and/or amelia as well as congenital heart diseases (CHDs). Other sporadic deformities that were detected are malformations of the inner and outer ear, and ocular abnormalities [2]. The burden on the affected children and their families pushed the health systems in Europe to issue new regulations and laws that control drugs’ usage and introduce checkpoints in clinical trials to ensure the approval process for novel drugs [3, 4]. Despite all these changes that occurred over the years, ethical considerations are sometimes still overridden at the crossroads of identifying novel drugs for urgent, severe, chronic, and life-threatening cases which keeps pharmaceutical companies with an upper hand in decision-making [5]. As for thalidomide, and nearly half a century after its withdrawal, it came back to the market as an FDA approved treatment for erythema nodosum leprosum (ENL) and multiple myeloma (MM) based on its anti-angiogenic potentials [4, 6, 7]. The usage of thalidomide and later its derivatives is now strictly controlled as all patients that are prescribed for these drugs must be registered on the System for thalidomide Education and Prescribing Safety (STEPS) program [8]. This is mainly because the exact mechanism by which thalidomide triggers its previously documented devastating effects, and its recent protective/healing effects is still elusive despite...
numerous publications on its effect on cell proliferation, DNA replication, transcription, synthesis and/or function of growth factors, synthesis and/or function of integrins, angiogenesis, chondrogenesis, and cell death [9]. Although there is no confirmed evidence that its therapeutic efficacy in MM and ENL is due to the direct inhibition of angiogenesis through modulating the expression of angiogenic molecules, yet this mechanism is among the most accepted ones [7, 10]. This also raises a possibility that the supposed anti-angiogenic effect of thalidomide is secondary to other unknown activity that can be context dependent. Nevertheless, the positive results on angiogenesis, have prompted many researchers to explore its usage alone or in conjunction with other drugs as an anticancer agent. Phase 2 of clinical trials is under way for many types of cancer including breast, ovarian, neck, hepatocellular and prostate carcinoma. We hereby review the proposed mechanisms of action of thalidomide and attribute its failure in treating lung cancer to the absence of its selective targets like members of the TBX2 subfamily in that context. We will focus also on the usefulness of genetic, chemical, and animal models to address the potential physiological and pathological modulatory effects of the newly developed drugs.

**Thalidomide: Structural/Functional properties**

Thalidomide is a chiral molecule and its teratogenic effect was linked to the usage of an equal racemic mixture of its two enantiomers: S-(−) and R-(+). It was demonstrated that at least in mice, the S-(−) isomer is responsible for the devastating effect. This simplistic view is challenged however by the chiral properties of many molecules, and this is the case of thalidomide which undergoes spontaneous interconversion between its 2 enantiomers under physiological conditions (▶ Fig. 1). The enantiomeric purity and chirality of drugs and their metabolites have taken a major spotlight in the pharmaceutical industry since then. This was even extended to the world of achirality, where recent advances showed how chiral molecules can impose their status on minerals [11]. Nonetheless the chirality of thalidomide was not its only disadvantage, but its lipophilic structure which makes it insoluble in water prevented it to be used intra-venally. However, this wasn’t the biggest challenge for scientists at the time when thalidomide was introduced as a sedative drug and even up till now, but it was the failing to recapitulate its teratogenic effect on rodent models. Thus, the reason why a clinical approval was “hastily” granted in the 1950’s for this drug was its safe usage on mice. This remains an enigmatic question despite “philosophical” attempts that link it to differential pharmacokinetics and/or oxygen reactive species, etc... [12, 13] That’s why with its reemergence as a potential anti-inflammatory and/or anti-angiogenic drug, many attempts have been made to synthetize analogs for thalidomide like lenalidomide, pomalidomide, and apermilast which are now referred to as immunomodulatory imide drugs (IMiDs). Those, in addition to thalidomide are now heavily used in treating ENL and MM [14].

**Erythema nodosum leprosum (ENL) and multiple myeloma (MM): common and divergent pathways**

The 1998 FDA approval for the usage of thalidomide in ENL treatment was a turning point in the drug historical saga as the underlying mechanisms by which thalidomide and those IMiDs are efficiently slowing the disease are not clearly understood. ENL is an inflammatory complication of leprosy through an obscure immunopathologic pathway which involves activation and/or repression of numerous cytokines amongst which are the tumor necrosis factor alpha (TNFα). It was shown that thalidomide and its derivatives can inhibit significantly post-transcriptionally and post-translationally TNFα in vitro through degrading its mRNA. [7, 10, 15]. On the other hand, clinical data from patients treated with IMiDs showed also a preferential transient but significant increase in other cytokines like interleukin-2 (IL-2), and interferon-gamma (IFNγ) boosting thus the overall immune system. The effect on TNFα seems to be mostly contradicta-
ble due to its dependency on the stimulus and the cellular context were thalidomide was shown to either enhance or decrease its synthesis/production. Nevertheless, the beneficial effect of this drug over the standard corticosteroids’ treatment moved thalidomide to be the preferential prescribed molecule despite its underscored neuropathy-associated adverse effects. Taken its beneficial impact on the immune-system and its potential anti-angiogenic effect as highlighted in different studies, the focus shifted rapidly over the last decade on its potential use in oncology. The FDA approved protocol for thalidomide in treating multiple myeloma in 2006 has paved the way for multiple clinical trials to start on various types of oncology, including solid tumors [9, 14, 16]. Again, despite the largely beneficial effects underscored by the different IMiDs, the exact mechanisms by which they helped improved the lives of those MM patients are still elusive. Based on the in vitro results, the major pathways involved in IMiDs’ therapeutical properties are linked to their anti-proliferative (FGF2), anti-angiogenic (VEGF), and down regulation of crucial cytokines (TNF-α, IL-6, IL-8). The drugs do also modulate expression of cell surface adhesion molecules including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) thus potentially altering the multiple myeloma- bone marrow stem cell interaction. Finally, thalidomide can potentially enhance the immune-system microenvironment through upregulating IL-2 and IFN which in turn boosts T-cells and natural killer cells. As in the case of ENL, the correlation of these in vitro evidence with in vivo clinical quantitated data is however still to be demonstrated in order to define whether they are relevant and/or specific.

**CRBN: general ubiquitination versus selective organ specific phenotype**

Indeed, the breakthrough in understanding Thalidomide’s teratogenic activities happened in 2010 with the identification of cereblon (CRBN) as the hit and lock protein for this drug [17]. Cereblon owes its name to its implication in brain development and to its LON domain (N-terminal La AT- dependent protease family). It is part of an evolutionary conserved family of proteins that exist across kingdoms except in Fungi. It was shown to bind as a cofactor for the damaged DNA-binding protein 1 (DDB1), which acts as the central component of an E3 ubiquitin ligase complex and regulates the selective degradation of key proteins in DNA repair, replication and transcription. The interface of CRBN-thalidomide binding was mapped to the C-terminal part of the protein (aa 317–428) referred to as the CULT domain (cereblon domain of unknown activity binding cellular ligands and thalidomide) (Fig. 1b). CRBN binds readily to thalidomide in vitro and to all other IMiDs with differential affinities and subsequently differential functions [18, 19]. Loss of function experiments in zebrafish recapitulate the exact effect of thalidomide in embryogenesis mainly affecting pectoral and ocular. It is hypothesized that in humans, thalidomide binds to the CRBN-DDB1 complex and inhibits proteasomal degradation of key factors involved in regulation like fibroblast growth factor 8/10 (FGF8/10) mainly in the limbs. Additional studies on lenalidomide have concluded that the proteasomal activity could be either altered positively or negatively making it once again difficult to correlate in vivo clinical activities to in vitro studies. Notably it was shown that pomalidomide accelerates proteasomal degradation of the key transcription Ikaros Zinc Finger Proteins 1 and 3 (IKZF1/IKZF3) that are significantly overexpressed in MM. These results challenge the validity of the systems being used as well as the approach [20]. Again, key questions arise from the use of the animal model and the relevance of human genetic data. On the other hand, the CRBN gene doesn’t play a key role in mouse development except for a mild behavioral phenotype. A well-documented genetic phenotype observed in humans with a p.R417X mutation in CRBN was associated with only mild form of mental retardation with no phenotype reminiscent of thalidomide teratogenicity (OMIN# 607417), thus questioning its specificity of being the targeted culprit gene.

**TBX2 Subfamily: a redundant function and a potential common target for thalidomide**

Among the newest reported potential targeted genes for thalidomide that aimed to explain its teratogenicity was T-box5 (TBX5). The T-box family of transcription factors to which TBX5 belongs is an evolutionary conserved family mainly involved in organ specification, and cellular proliferation and differentiation. Members in this family share a highly conserved 180 amino acids referred to as the T-box domain through which they exert their transcriptional modulation of gene expression (Smith, 1997; Packham and Brook, 2003). Based on amino acid sequence homology in this domain and their tissue expression specificity; these members are further subclassified into categories. The TBX2 subfamily is composed of 4 members (TBX2,3,4, and 5) which are co-expressed in many organs yet with distinct and only slightly overlapping functional pattern. In nearly all experimental animal models, the loss of one allele of TBX5 recapitulate the Holt-Oram syndrome (HOS) in humans which is caused by various mutations in the same gene [21] Interestingly, HOS patients have phenotype that overlap with those observed in children whose mothers were exposed to thalidomide during pregnancy. This similarity between the phenotypes allowed for a contextual relevant bases that links thalidomide effect to the TBX5 gene which was further elaborated through in silico docking experiments coupled to in vitro biochemical studies [33]. The later showed that thalidomide binds specifically to TBX5 protein in its T-box domain obliterating its specific interaction with the DNA (Fig. 1c). Recently, the use of thalidomide on chick embryos was shown to directly affect the expression of TBX5 providing an in vivo evidence for the effect of the drug on TBX5. Although the role of TBX2 subfamily is mainly studied in the heart and the limbs, yet these genes were reported to be highly and preferably expressed in the lungs were all the 4 members are expressed in embryonic and postnatal stages in both mice and humans [22]. In mice that lack both alleles of Tbx2 (Tbx2cre) mice, Tbx2 was shown to be essential in controlling lung growth by restricting cell proliferation and inhibiting lung mesenchyme differentiation [23, 24]. Additionally, lungs from Tbx2-deficient embryos were hemorrhagic, visibly smaller and with reduced branching. Of note, lung branching is dependent on functional interaction between Tbx2 and Tbx3 with both genes functioning redundantly to preserve branching morphogenesis. Depletion of Tbx4 or Tbx5 by antisense oligonucleotides was shown to hamper bronchial differentiation in ex-vivo lung cultures. Targeted inducible inactivation of Tbx5, but not of Tbx4, was demonstrated to inhibit lung bud and tracheal formation [25, 26]. In parallel, we have recently showed a preferential expres-
Expression levels of *TBX2*, *TBX3*, *TBX4* and *TBX5* mRNAs were analyzed in > 7,000 pan-normal specimens using the Genotype Tissue Expression Project (GTEx) and the results showed consistent high expression of these genes as is the case in the mouse. Interestingly all four members were consistently and significantly downregulated in lung adenocarcinomas suggesting that they might play an essential role in lung tumorigenesis. A role that is even more highlighted by the early suppression of their expression in the lung field of cancerization and even before development of the tumors. Adding to this was the apoptotic effect detected after transfecting different lung adenocarcinoma (LUAD) cell lines with members of TBX2 subfamily [27]. Taking into consideration the expression profile of the Tbx2 subfamily members in lung cancer and their shared high homology, as well as the direct interaction of the TBX5 protein with thalidomide, we predicted that treating lung cancer with thalidomide is going to fail.

**Lung Cancer: the failure of thalidomide due to the absence of its target?**

As mentioned previously, the usage of thalidomide in treating multiple myeloma has opened the way for its potential application in many cancers including lung cancer. Needless to say that lung cancer is still the leading cause of death amongst all cancer types as it occupies the first place in the number of new cases of cancer in most of the countries [28, 29]. It was thus one of those hotspots for early clinical trials to start combining thalidomide with ongoing regimens. The backbone for such an enthusiasm was derived from early studies using human lung cancer cell lines were thalidomide was shown to inhibit cellular proliferation and to induce apoptosis. Those experiments were however done on only few types of lung cancer cell lines that do not include lung adenocarcinomas and only one dose of thalidomide was used. On the contrary, our ongoing studies [27] show that in LUAD cell lines like NCI-1944 which lack the expression of all Tbx2 subfamily members, cellular proliferation increased by 40% after 48 h of treatment with the lowest concentration of thalidomide as compared to vehicle treatment with DMSO (▶ Fig. 2a). A more prominent effect was detected in transformed human bronchial epithelial cells (HBEC-2) which slightly express TBX5 cells and reached 60% increase in proliferation at the same conditions (▶ Fig. 2a). However, and in conditions where growth factors were not supplemented in cultured media, i.e. lack of serum, NCI-H1944 cells showed dramatic suppression of cell growth after 24 and 48 h of treatment. On the other hand, HBEC-2 cells showed a transient suppression (after 24h) followed by tremendous increase (80%) in their proliferation potential after 48 h of treatment with thalidomide (▶ Fig. 2b). These *in vitro* experiments are in line with the failure of phase III clinical trials of lung cancer conducted over the last 5 years. In the first large trial of patients with NSCLC, thalidomide was combined with gemcitabine and carboplatin chemotherapy and 722 patients were followed up for 2 years under a randomized double-blind placebo-control design [30, 31]. The disappointing end result was that thalidomide didn’t improve survival overall, while it increased the risk of throm-

![Fig. 2](effect-of-thalidomide-on-hbec-2-and-nci-h1944-viability-in-serum-enriched-a-or-depleted-b-media-cell-numbers-in-each-well-were-assessed-using-the-mtt-assay-the-absorbance-at-570-nm-corresponding-to-the-cells-treated-with-dms-0-3-was-defined-as-100-data-are-presented-as-mean-standard-deviation-with-significance-compared-to-dms-treatment-p<0.05)
Fig. 3  Differential Expression of Genes Involved in Thalidomide’s Activities. The Gepia online console (gepia.cancer-pku.cn) was used to draw boxplots (Log2 scale) for the differentially expressed mRNA between 483 tumor samples (red) and 347 control and TCGA normal samples (grey). The expression differences were statistically analyzed using Wilcoxon rank sum tests. Boxes represent 25–75% ranges. Solid horizontal lines represent median values with * p < 0.01. 

a TBX2 subfamily. 

b Angiogenic factors and their receptors. 

c Cereblon and its targets Ikaros Zinc Finger Proteins. 

d Immuno-modulated genes and adhesion molecules.
botic events. More importantly, the survival was significantly worse in patients with non-squamous histology, mainly LUADs. This trial before the CRBN-thalidomide era was based on the potential use of thalidomide as anti-angiogenic factor targeting both VEGF and FGF2 that are usually overexpressed in cancer. Interestingly we surveyed the expression of VEGF, FGF2, and their receptors in LUADS using the GEPIA-cancer console, and found that the expression of these factors is not altered and even slightly repressed versus the control. In particular the FGF receptor is significantly repressed ($p < 0.01$) paralleling the downregulation of all TBX2 family members ($\text{▶ Fig. 3a, b}$). These results support our hypothesis that in the absence of TBX5 and the other potential targets, one should not expect any positive effect for thalidomide treatment in lung cancer.

The second large Phase III study was done in the same way but with additionally combining of radiotherapy on stages III and IV patients with a total 546 enrolled patients [32]. The end results showed increased toxicities by thalidomide that prompted the premature arrest of the trial. Moreover, the assessment of survival rate showed that thalidomide did not improve in those patients with locally advanced NSCLC. Despite being conducted between 2002 and 2006, the results of this trial were published in 2012, after the publications of the CRBN-thalidomide interaction. To avoid any hastily conclusions concerning the role of TBX2 family members in this resistance towards thalidomide, we surveyed the expression of CRBN and its targets in multiple myeloma (IKZF1/3) which showed that none is overexpressed in LUADs and thus couldn’t be a target for the treatment ($\text{▶ Fig. 3c}$). We further eliminated all other potentially regulated targets by thalidomide by showing that none of them is altered in LUADs, whether cytokines (TNF, IL, IFN) or adhesion molecules (ICAM, VCAM) ($\text{▶ Fig. 3d}$).

Therefore, the absence of TBX2–5 in lung cancer cells would impair the potential activity of thalidomide through its bona fide targets and thus would not stop the proliferation of these cells as desired ($\text{▶ Fig. 4}$). While in the above both clinical settings, we hypothesize that an inhibition of TBX2–5 activity by thalidomide would mimic their downregulation and put the normal epithelial cells at the mercy of being transformed into adenocarcinomas, thus worsening the situation of the patients instead of improving it.

**Conclusion**

Understanding the genetic/genomic context of diseases is essential in the new era of personalized medicine, and this is even more relevant for a devastating disease like lung cancer. The failure of clinical trials in most of the cases is attributed to the lack of knowledge of the molecular mechanisms underlying the mode of action of the drugs used or their metabolites. In the case of thalidomide and despite all the recent advancements in our understanding of its targeted entities, there are no relevant clinical data that match
the in vitro results and reveal its mode of action. In this perspective, we explained the failure of using thalidomide in clinical trials for treating lung adenocarcinoma based on the tumor suppressor role of TBX2 subfamily members coupled with our in vitro studies on the thalidomide/T-box interaction which abrogates their function. Thus, we postulate to survey the expression of these genes as well as the known novel targets of thalidomide in any context before conducting further trials on this drug. The call is finally for a synergistic effort between basic scientists, physicians, pharmaceuticals, and health systems to consolidate effort to ensure proper a priori knowledge of the mode of action and safety of a drug and its metabolites before starting clinical trials.

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Conflict of Interests

The authors declare no conflict of interests.

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